Influences of extrinsic factors on gene expression and selection of B-1 cells

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1 Introduction

1.1 The innate and adaptive immune system

In order to protect the body against the diverse challenges provoked by a huge array of potentially harmful organisms and substances in our environment, vertebrates during evolution have developed an elaborate network of cells and tightly regulated molecular mechanisms, that are commonly known as the immune system. Principally there are two major requirements this specialized system has to fulfill: First there has to be a fast reaction against each invader, that keeps the potential infections in check. Secondly, a specific and at the same time flexible reaction against the highly diverse nature of invaders has to take place in order to rid the body of harmful consequences.

To accomplish this, two different branches of the immune system have evolved which act in concert in a highly efficient way. They are referred as the innate and the adaptive immune system, respectively. The former represents an early activated, rapidly reacting “first line of defense”. It comprises effector cells and molecules that respond rather against common molecular patterns than specific structures, for instance invariant pattern recognition receptors can recognize bacterial cell wall components or double-stranded RNA during infection cycles of viruses (Mogensen and Paludan, 2005). The innate immune system is capable to repel most of the attacks in daily life so efficiently that we often do not even notice them. However, some organisms - therefore called pathogens - have acquired the ability to overcome this first barrier and to spread into the tissues or blood system of their hosts. Nevertheless, these pathogens are still kept in check by effector mechanisms of the innate immune system as mentioned above. At the same time the adaptive immune system comes into play. The corresponding cells recognize pathogens by means of highly specific receptors. Additionally, this branch of the immune system mediates a sometimes lifelong protection against re-infection based on an immunological memory.

1.2 B and T lymphocytes - players of the adaptive immune system

The defense mechanisms of the adaptive immune system are based on the functionality of two cell types: Thymus derived T lymphocytes, and B lymphocytes, originally named after the organ their development was discovered first: the Bursa of Fabricius in birds. In most vertebrates like mice and man the main site of B-cell development is the bone marrow.

T as well as B lymphocytes, both have the inherent capability to develop highly specific surface receptors against antigens (antigens are defined as molecules or structures, that induce an immune response). Importantly, individual lymphocytes express exclusively one receptor type against one specific antigenic determinant (called epitope). Upon antigen encounter of the specific antigen the respective clones react by clonal expansion and differentiation.

Generally T and B lymphocytes differ in their way to mediate protection. T lymphocytes which derive from hematopoietic stem cells and develop from precursors after their migration to the thymus, mediate so called cellular immunity - reflecting the fact, that their effector function is mediated via cell contacts. The major group of T cells cannot recognize their respective antigens directly. Instead, they have to contact antigen-presenting cells that provide the respective antigens in a processed form. For this purpose the latter bear molecules on their surface encoded in the major histocompatibility complex (MHC) that accommodate small peptid fragments of the antigen processed within these cells. There are two types of MHC molecules: class I and class II. MHC class I molecules are found on the surface of almost every nucleus containing cell. They present antigens that are derived from the cytosol of the cells e.g. during a viral infection (Hofmann et al., 2001). MHC class II molecules represent the second class of MHC molecules, solely found on the surface of so called professional antigen-presenting cells, i.e. macrophages, dendritic cells and B cells (Bryant and Ploegh, 2004). They present fragments from antigens that have been taken up from the surrounding environment by pinocytosis or that has gained access to intracellular vesicles by phagocytosis. B cells accumulate antigen via their specific B cell receptor and thus provide the basis for specific T cell help by presenting it via MHC class II (Parker, 1993).

For antigen recognition, the T cell receptor (TCR) binds to the antigen:MHC complex. However, in order to fully activate the lymphocyte function further co-stimulatory signals are needed. This is provided by either the CD4 or the CD8 co-receptor. Mature T cells of healthy individuals bear exclusively one of these co-receptors and T cells expressing either of them exhibit different effector functions. Thus, their surface expression was used to further classify the T cell compartment into two major subsets: the $CD8^+$ cytotoxic T cells and the $CD4^+$ T helper cells (Woodland and Dutton, 2003). While the former can identify and kill intracellularly infected host cells (Wong and Pamer, 2003), the latter are specialized for the activation of other cells of the immune system, e.g. macrophages or B cells, leading to stimulation or inhibition of immune reactions (summarized in Woodland and Dutton, 2003).

B lymphocytes (or B cells), as the second column of the adaptive immune system are responsible for the humoral immunity with antibodies as effector molecules (see also 1.4.1). These immunoglobulins (Ig) can occur in two physical forms, a soluble (secreted) and a membrane bound. The membrane bound form acts as the antigen-specific B cell receptor (BCR) on the surface of B cells.

The BCR is composed of an antibody molecule that is equipped with an membrane integral C-terminal peptide. It is associated with the Igα/Igβ heterodimer, which functions as a signal transduction unit. This heterodimer connects the BCR to an organized complex of cytoplasmic signaling molecules. On mature B cells the BCR provides either a maintenance signal, required for the long-term survival of these cells (Lam et al., 1997), or an activation signal upon exposure to antigen (Gauld et al., 2002).

If a B cell becomes activated by antigen - either by crosslinking of the BCR or by the disturbance of an ordered oligomeric structure of BCR-complexes as recently proposed by Reth (Reth, 2001) - it starts to differentiate and might develop into a plasma cell. The sole function of plasma cells is the secretion of antibodies thereby abolishing the surface expression of the BCR (Calame et al., 2003). In the secreted form the membrane integral and cytoplasmic domains of the BCR are replaced by a secretory tail due to alternative splicing (Ross et al., 1998). B cells can in addition modulate the binding features and effector functions of their immunoglobulins by class switch (see 1.4.5) and/or somatic hypermutation (see 1.4.4) before plasma cell differentiation (summarized in McHeyzer-Williams, 2003).

As mentioned already antibodies are the main effector molecules of the humoral immune response. In contrast to the T cell receptor antibodies recognize native antigens present in body fluids and cavities. For instance, they can bind bacterial toxins or viral particles thus blocking their access to cells (neutralization). Additionally, antibody-antigen complexes influence the inflammatory response and cell-mediated immunity indirectly by either complement activation or cross-linking of Fc-receptors thus promoting phagocytosis (e.g. by macrophages or neutrophils). This may lead to further stimulation of the immune system by production of cytokines, chemokines and other inflammatory mediators. Finally, there are many examples of antibodies which promote direct anti-microbial activities, e.g. by blocking the function of physiological relevant molecules of pathogens (Casadevall and Pirofski, 2004).

Taken together B cells and antibodies produced by them provide a huge impact on the protective immunity against viral, bacterial and parasitic infections. Their presence after a first encounter with antigen by infection or vaccination might completely abolish reinfection by the particular pathogen.

1.3 B cell subsets

So far B cells have been described as a homogenous group of immune cells. However, these lymphocytes are much more heterogeneous to accomplish protection at different stages and time points of an infection. On the basis of phenotypic, topographic and functional characteristics, two major subsets of mature B cells have been described, which have different requirements for their generation and maintenance (Herzenberg, 2000).

The first subset, termed B-1 cells, are enriched in the peritoneal and pleural cavities where they persist by self-renewal as their development takes place mainly during early ontogeny: B-1 cell progenitors are found within the embryonic splanchnopleura as well as later in the fetal omentum and liver. Their development terminates shortly after birth (Herzenberg, 2000).

B-1 cells are believed to be the primary source of natural serum IgM antibodies. These antibodies arise in the absence of exogenous antigenic stimulation (Bos et al., 1988; Haury et al., 1997) and often display polyreactive, weakly autoreactive binding capacity as well as reactivity against many common pathogen-associated

carbohydrate antigens (Baumgarth et al., 2005). According to the expression characteristics concerning the surface expression of CD5, the B-1 cell pool is further divided into two subgroups: $CD5^+$ B-1a cells and $CD5^-$ B-1b cells. Except for the spleen where B-1a cells comprise a 1-5% population of B cells (their absolute number is similar to the number of B-1 a cells found in the peritoneum (Kantor et al., 1992)), B-1 cells are commonly not found in the other peripheral lymphoid organs.
In contrast, the second group of mature B cells termed B-2 or conventional B cells represent the predominating B-cell population in the peripheral lymphoid organs including spleen and are the source for monospecific antibodies. B-2 cells are the essential players of the adaptive B cell immune response. Although precursors of B-2 cells can be already detected in the fetal liver their main site of generation is the bone marrow (Herzenberg, 2000; Berland and Wortis, 2002). From this source, newly formed B-2 cells continuously enter the mature B-cell pool throughout life. Like the B-1 population, also the B-2 population shows some degree of heterogeneity and therefore can be further divided into subpopulations. Besides the above mentioned recirculating follicular B cells (FO B cells) which locate in the B-lymphoid follicles within spleen or lymph nodes, marginal zone B cells (MZ B cells) exist as a splenic subset of B-2 cells (Lopes-Carvalho and Kearney, 2004). They are named after the marginal zone where they locate as resident cells. This specialized microenvironment is positioned at the periphery of the periarterial lymphatic sheath (PALS) and the follicular area, at the border between the white and the red pulp. Here, arterial blood empties into open sinuses. Predestined by their exposed location to the blood stream, MZ B cells are adapted to react quickly to blood-borne pathogens (Martin and Kearney, 2001; Lopes-Carvalho and Kearney, 2004; Allman et al., 2004). By providing fast antibody responses as well as interaction with T cells this B cell subset thus helps to bridge the temporal gap between the innate and adaptive immune response.

1.4 Antibodies and their generation

Antibodies as the effector molecules of B cells contribute in different ways to an immune response. Thus, they cannot not only bind specific antigens but also stimulate other parts of the immune system to participate in the immune response.

These important functions are mediated by different parts of the antibody molecule which are the focus of the next chapters.

1.4.1 Antibody structure

The antibody molecule is formed by two identical heavy (H) as well as two identical light (L) chain polypeptides which are shaped into the antibodies' particular structure by covalent (disulfide-bonds) and non-covalent interactions (Figure 1.1). Both chains can be divided into domains on the basis of sequence similarities. The amino-terminal domain of each chain is variable in sequence when several antibodies are compared. The remaining domains are constant.

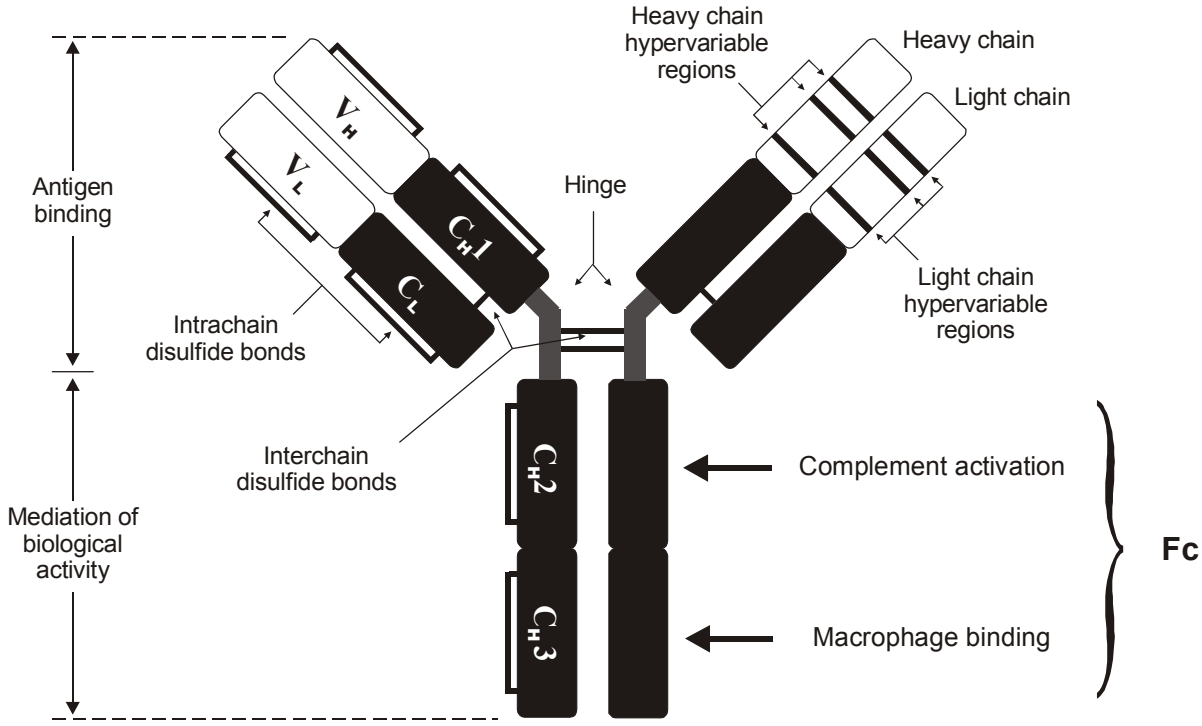


Figure 1.1. Schematic design of the IgG molecule domain structure. According to their constant (Fc) region of their heavy chains antibodies can belong to one of five isotype classes named IgM, IgD, IgG, IgA and IgE. The light chains can be either of the κ or λ isotype. The hinge region provides steric flexibility. It is only found in IgG, IgD and IgA whereas IgM and IgE contain an additional C_H domain instead.

The variable region of either chain is based on a structural backbone formed by four relatively invariant framework sequences. These are interspersed by three regions

termed hypervariable or complementarity determining regions (CDRs). On the three dimensional structure the CDRs of the heavy and light chain form the actual antigen binding site of an antibody. Accordingly, the CDRs display an extraordinary high variability, the highest diversity always found in the CDR3 regions. The V regions of the heavy and light chains are C-terminally followed by C-regions. The C-region of the heavy chain determines the class or isotype of an antibody. There are five main immunoglobulin classes known: IgM, IgD, IgG, IgE and IgA. Moreover, in mice the IgG class is further divided into four subclasses termed IgG1, IgG2a, IgG2b and IgG3. Corresponding to their class affiliation the heavy chains are designated by the according Greek letters: μ,δ,γ,α and ε (γ_1, γ_{2a}, γ_{2b}, γ_3, respectively). A major difference among heavy chains is the number of C domains. While μ and ε consist of four domains γ, δ and α chains display only three domains. However, in the latter the C domain 1 is linked with C domain 2 by a so called hinge region that provides flexibility to the antibody molecule. The more V-distal part of the C_H-region, also called Fc-region, decides about the functional characteristics of an antibody, such as its half-life in serum, activation of complement and the ability to interact with Fc-receptors (summarized in Frazer and Capra, 1999).

Of all existing classes and subclasses two are secreted as multimers. IgM can be found as penta- or hexamers whereas IgA usually forms dimers (Johansen et al., 2000). The multimers are assembled from monomers involving an additional polypeptide J (joining) chain. Because of the multimeric binding sites the overall binding strength (avidity) of the antibodies belonging to the multimer forming classes is dramatically increased. Therefore, even low affinity antibodies as often found among IgM antibodies become effective.

The two light chain types are termed κ or λ. In contrast to the constant parts of the heavy chains no effector properties have been linked to their C_L domains. Among different species the ratio between antibodies that bear light chains of κ or λ type varies considerably, e.g. in mice a ratio of 10:1, while in horse or cattle the opposite ratio is found. In humans the κ:λ ratio is 6:4 (Frazer and Capra, 1999).

Although the particular constant regions of heavy and light chains display very little sequence variability, polymorphisms can be found. These allelic variants are called allotypes. Antibodies against the allotypes of the heavy chain C-regions of mice from different inbred strains are valuable tools. For instance they can be taken to measure

antibodies derived of mixtures of B cells from different inbred strains. Thus they represent essential reagents for transfer experiments of cells or antibodies.

Mice of the BALB/c background, mainly used in this work are of the heavy chain allotype IgH^a, whereas C57BL/6 mice of another widely used lab strain produce IgH^b allotypic antibodies (Ovary, 1982). The CB20 strain used in this work is a BALB/c strain that carries the IgM^b allotype.

Antibodies can also be distinguished by their idiotypes (Id) (summarized in Greenspan and Bona, 1993). The idiotype consists of distinct antigenic determinants (idiotopes) found on the V regions of an individual antibody. The idiotope is always defined by a monoclonal antibody. There are private idiotopes that are associated with unique antibodies and public idiotopes (also termed cross-reactive idiotopes) that are shared by different antibody molecules.

Antibodies directed against such idiotopic determinants are termed anti-idiotypic antibodies (anti-Id). Some of them recognize conformational determinants dependent on combined V_H and V_L interactions, others are specific for either V_H or V_L chain determinants.

1.4.2 The immunoglobulin loci

The genes that encode these immunoglobulin molecules are located on different chromosomes. The heavy chain locus of mice resides on chromosome 12, while the κ and λ light chain loci are found on chromosomes 6 and 16, respectively. Unique to the immunoglobulin loci is that the gene segments that encode the variable regions of the BCR or later of the antibodies do not exist in a form that allows their direct transcription from DNA in germline configuration. Instead they have to be assembled from small gene segments during the ontogeny of a B cell. This is accomplished in a process involving the somatic rearrangement of DNA.

1.4.2.1 The heavy chain locus

The V-region of immunoglobulin heavy chain (IgH) genes is assembled from three different types of gene segments termed V_H, D_H and J_H. They are distributed over an estimated length of 1.5 megabases (Mb) (Figure 1.2). The exons encoding the constant parts of the heavy chains follow further downstream extending the whole locus to approximately 3 Mb (Chowdhury and Sen, 2004).

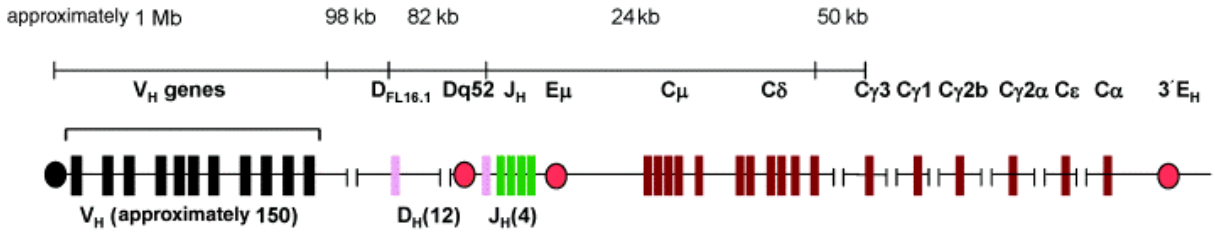


Figure 1.2. Immunoglobulin heavy chain (IgH) locus organization. A schematic representation of the murine IgH with approximate distances between the gene segments is shown. Several V_H genes (black rectangles), the 5'-most (DFL 16.1) and the 3'-most D_H gene segments (pink rectangles) are indicated along with the different constant region isotypes. Numbers of members belonging to each gene family are written in parentheses below the line. Three known cis-regulatory elements, the intronic enhancer E_μ, the Dq52 promoter, and the 3' locus control region are depicted as red ovals (Chowdhury and Sen, 2004).

More than 100 V region segments (V, D, J) have been classified in the mouse so far (Chevillard et al., 2002; Chowdhury and Sen, 2004). The V_H segments are assigned to 15 partially interspersed gene families. Close to half of the murine V_H genes belong to the J558 family, the majority being located at the 5' end of the V_H cluster. The extreme 3' end of the V_H locus comprises the 7183 family. An overview about the V_H gene family is given in Figure 1.3.

Besides the functional V_H gene segments a number of pseudogenes can be found, that are either not able to rearrange or lead to non-functional polypeptide chains due to mutations.

Downstream of the V_H gene segments a cluster of 13 D gene segments follows. The first 12 D gene segments are relatively evenly distributed over a 60 kb region, whereas the thirteenth most 3' D segment, Dq52, lies in much closer proximity to the J_H gene segments, separated from the other D members by a 18 kb gap. Its distance to the nearest J_H gene segment (J_H1) of the following J cluster adds up to 1 kb. The J cluster consists of 4 functional gene segments.

The first exons encoding the constant part of the heavy chain follow further downstream. C_μ and C_δ are located within the next 12 kb. After a gap of 50 kb the exons for the different subclasses of γ chains are found followed by C_ε and finally C_α (Figure 1.2) (Chowdhury and Sen, 2004).

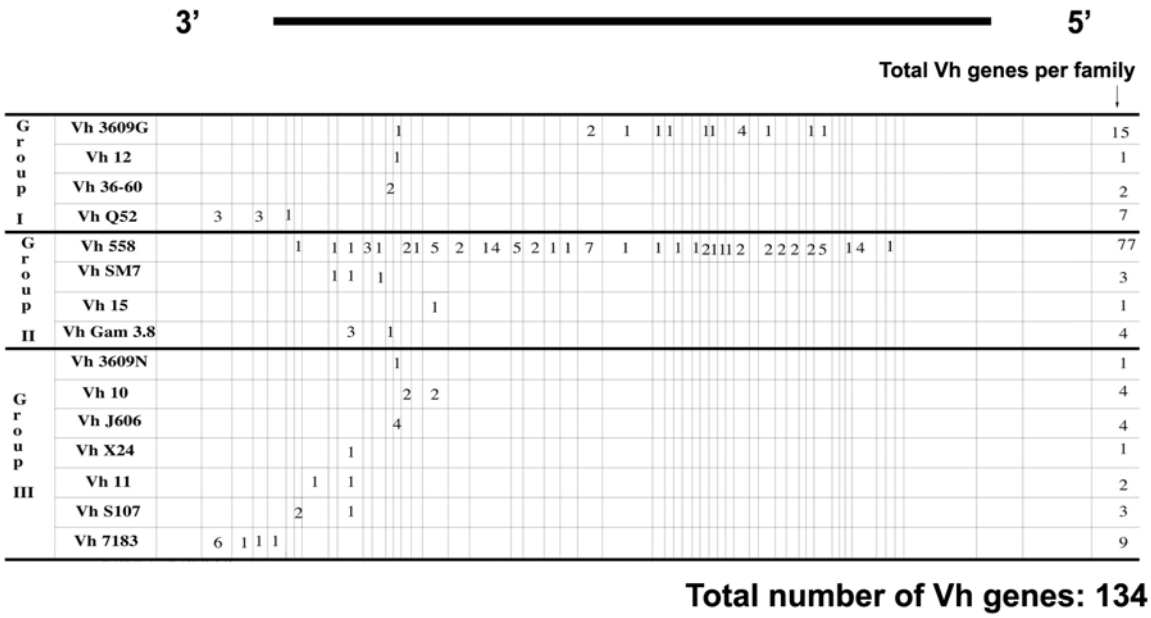


Figure 1.3. Schematic representation of the murine V_H locus. The map is shown in chromosomal orientation, left is toward the centromer, this is opposite to the transcriptional orientation. The relative chromosomal location of respective family members is given as well their total number. Pseudogenes have not been included (Graph modified after Chevillard et al., 2002).

1.4.2.2 The light chain loci

Both types of light chains, κ and λ, appear to be functionally identical. Only their order of expression during ontogeny appears to differ (Engel et al., 1999; Duber et al., 2003).

The κ locus

The murine Vκ-locus (Figure 1.4) consists of 140 localized and sequenced Vκ gene segments. They can be classified into 75 functional, 21 potentially functional and 44 pseudogenes and spread over 3 Mb (Roschenthaler et al., 2000). Like in the case of the V_H genes, different Vκ gene segments can be assorted due to sequence homologies into 18 different families. Particular for this cluster is that Vκ segments can be found in different transcriptional orientation. Upon rearrangement this leads either to the deletion of intervening Vκ segments or to their inversion (summarized in Schlissel, 2004).

Further downstream the Vκ cluster is followed by a set of five Jκ segments, one of which (Jκ3) is a pseudogene. A single exon for the constant region finally locates 2.5 kb 3' of the Jκ cluster.

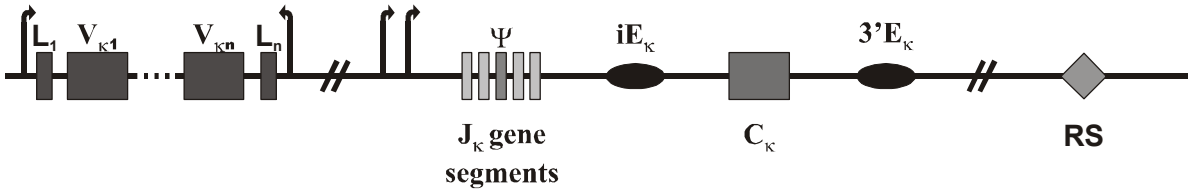


Figure 1.4. The murine κ light chain locus. Gene segments are shown in germline configuration (not to scale). One peculiarity about the Vκ locus is indicated: Its gene segments are located in both transcriptional orientations respective to the C region. Arrows indicate promoters and transcription direction. The black ovals represent the intron enhancer iEκ and the 3' enhancer 3'Eκ, both promoting transcription of the rearranged light chains. Pseudogenes are designated by Ψ and RS indicates the recombining sequence that can mediate Cκ deletion. (Figure adapted from Düber, 2004).

The λ locus

In contrast to the κ locus with its multiple V-region families, the Vλ region (Figure 1.5) includes only three V-segments, termed Vλ1, Vλ2 and VλX. Also different from the organization of the heavy or κ chain locus, the λ-locus harbors, instead of the spatially separated unique J or C regions described earlier, four gene clusters, each containing one J and one C segment, respectively (Figure 1.5). From these four clusters, however, one (Jλ4-Cλ4) is nonfunctional due to a frame shift in the C region, defective recombination signal sequences as well as a defective RNA donor splice site (Weiss and Wu, 1987).

The J-Cλ3 and J-Cλ1 genes are arranged in one cluster about 3 kb apart with the Vλ1 gene lying about 16 kb upstream of J-Cλ3. The second Cλ sequence cluster is positioned about 130 kb upstream of the Cλ3 locus and contains J-Cλ2 and the unexpressed J-Cλ4-combination followed by the two Vλ genes Vλ2 and VλX further upstream. VλX however is only rarely used as it has an in-frame termination codon at its 3' end (Gerdes and Wabl, 2002).

The gene order (V2-Vx-CJ2-JC4-V1-JC3-JC1) explains why Vλ2 (or sometimes VλX) is commonly found in association with Cλ2 whereas Vλ1 combines with Cλ1 or Cλ3. In rare cases Vλ2 has also been found in association with the 190 kb distant Cλ1 and Cλ3, however backward recombination of Vλ1 with Cλ2 has not been observed so far (Weiss et al., 1985).

The λ1 gene was the first mammalian single copy gene ever to be isolated and sequenced (Tonegawa et al., 1978a; Tonegawa et al., 1978b), subsequentially followed by the others (Arp et al., 1982; Blomberg and Tonegawa, 1982; Miller et al., 1982; Selsing et al., 1982; Weiss et al., 1985; Weiss and Wu, 1987; Sanchez et al., 1990).

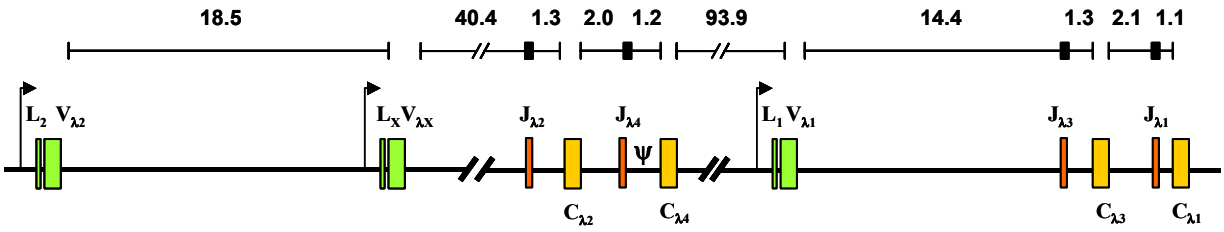


Figure 1.5. Physical map of the mouse λ light chain locus. Picture of the genomic organization of the various λ gene segments. The distances are given in kilobases (kb). Transcriptional orientation is from left to right (promoters illustrated by arrows). ψ indicates the pseudogene. (Modified after Gerdes and Wabl, 2002).

1.4.3 The rearrangement process

The rearrangement process that underlies the assembly of the B cell receptor gene segments explains the capability of B cells to recognize a tremendous number of different antigenic determinants. Because it involves the genomic relocalization of the different types of variable gene segments it is called VDJ rearrangement in the case of the heavy chain and VJ rearrangement in the case of the light chains, respectively. However, receptor rearrangement is not restricted to B cells. T cells, that have to fulfill equal requirements regarding antigen recognition, use the same mechanism and - as described later in this chapter - even the same machinery for it (for review

see Livak, 2004). The genes encoding the T cell receptor are however different from the BCR genes.

In both B and T cells the assembly of the V regions to functional transcription units is a highly ordered process. Nevertheless, it includes some volitional imprecisions that contribute to further diversity in addition to the combinatorial diversity that is achieved by the combination of different gene segments.

The assembly of the heavy chain V-region in the mouse starts with the combination of one of the D-segments with one of the four J-segments (Figure 1.6). This first step is then followed by the connection of one of the V genes to the newly formed D-J sequence, leading to a final VDJ-sequence encoding the entire V-domain (Chowdhury and Sen, 2004). The connection with the constant part of the immunoglobulin is then later on achieved by RNA splicing of the primary transcripts.

The recombination process is mechanistically very similar for heavy and light chain genes (Jung and Alt, 2004). Differences occur mainly due to the lack of D segment recombination step in the light chains.

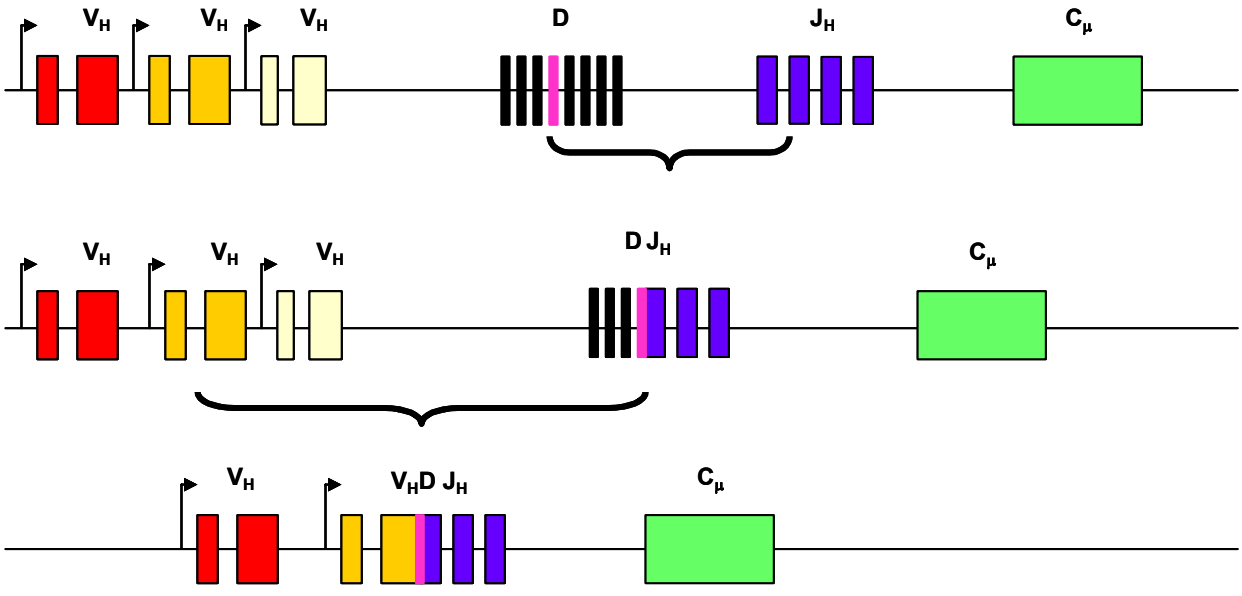


Figure 1.6. Schematic illustration of heavy chain rearrangement. A D segment is first combined with a J segment followed by addition to a V segment. After the final VDJ recombination step transcription can occur. Remaining J segments of the recombination process are removed by splicing of the primary transcript thereafter (promoters illustrated by arrows).

The correct order of V(D)J assemblies (first V, then (optionally) D followed by J) is dependent on short, conserved, non coding DNA sequences flanking each of the single gene segments (Jung and Alt, 2004). They are known as recombination signal (RS) sequences and serve as recognition sites for the recombinase machinery. RSs consist of relatively conserved heptamers and nonamers, with respective consensus sequences of CACAGTG and ACAAAAACC. They are separated by a non-conserved spacer of either 12 or 23 basepairs (Figure 1.7).

V_H and J_H segments are both flanked with RSs with 23-bp spacers (here termed as 23RS), while D_H segments are flanked on either side with RSs containing spacers of 12 bp (12RSs).

At the light chain loci V_κ segments are followed by 12RSs whereas J_κ segments are flanked with 23RSs while for the Igλ locus the reverse is found. Recombination occurs only between one RS with a 12-bp spacer and one with a 23-bp spacer thus directing recombination between appropriate gene segments. The underlying principle is referred to as the "12/23" rule (Early et al., 1980).

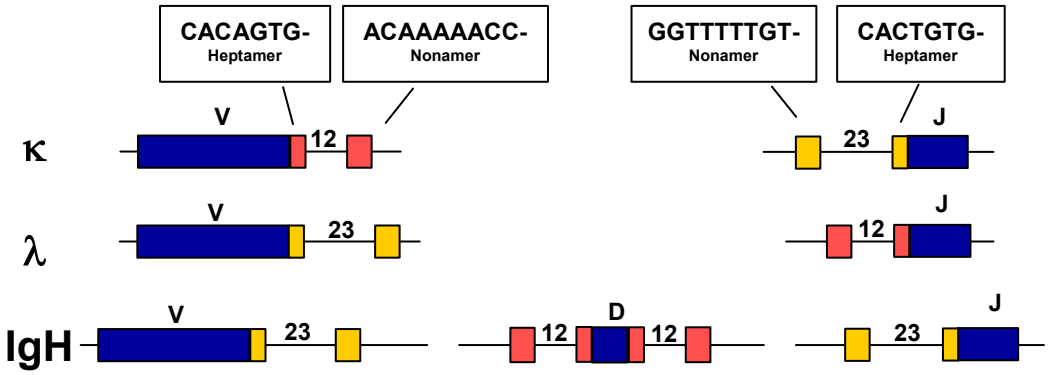


Figure 1.7. RSs at the different loci. Depending on the locus, V segments may be flanked by 12- or 23-bp RSs, and similar for J segments. But one of each type of element must be present for recombination to occur, a requirement that prevents futile recombination events (e.g. J to J).

The recombination events are mediated by the recombinase machinery, the process being started by the gene products of the recombination activating genes *Rag1* and *Rag2*. Both are indispensable for V(D)J recombination as *Rag1* or *Rag2* deficient animals exhibit a complete lack of mature B and T cells (Mombaerts et al., 1992;

Shinkai et al., 1992). The RAG-proteins form a complex (RAG) providing the endonuclease activity for the formation of double strand breaks (DSB) that are necessary for the DNA rearrangement. To initiate the cleavage reaction, RAG binds first to one (12 or 23) and then to a second (23 or 12) RS introducing a precise nick at the very border of each RS and the particular gene segment.

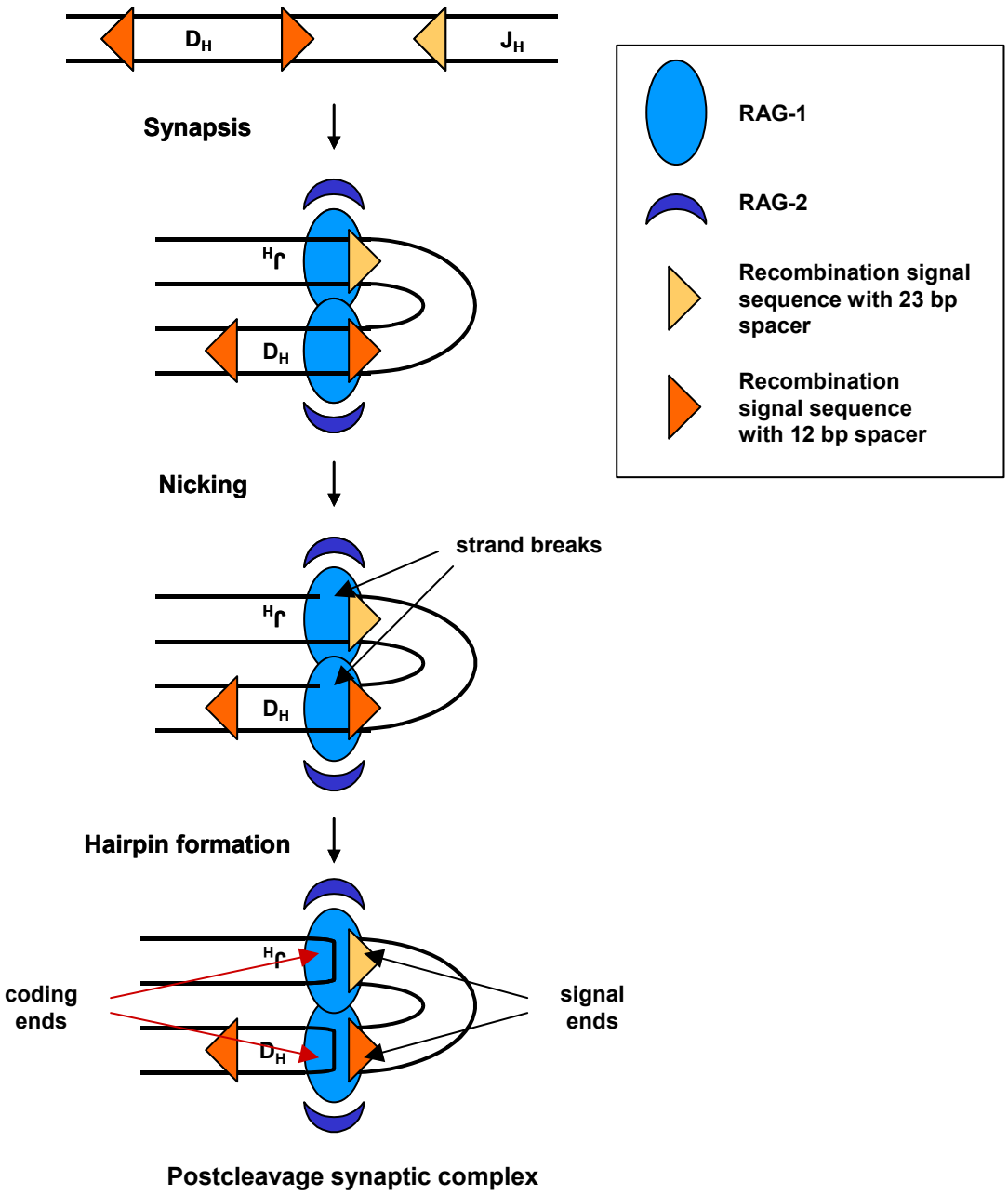


Figure 1.8. Summary of the RAG cleavage reaction (Jung and Alt, 2004).

After this initiation step the resulting free 3'-OH end on the coding strand generates a blunt, 5'-phosphorylated RS end and a closed hairpined coding end through direct nucleophilic attack on the opposite phosphodiester bond (Figure 1.8). The four free DNA ends remain associated with RAG in a post-cleavage synaptic complex, two belonging to the coding strand and two belonging to the ends of the RSs, respectively. The postcleavage synaptic complex has basically two functions: to protect the DNA ends from inappropriate insertion elsewhere in the genome and to recruit DNA repair enzymes that are supposed to catalyze the following DNA religation steps (Jung and Alt, 2004; De et al., 2004).

There are currently six identified repair proteins known to be involved in this step. They can be subdivided into two classes. One consists of the proteins Ku70 and Ku80, which form a DNA binding heterodimer, and XRCC4 and DNA ligase 4, which form an end-ligation complex (Bassing et al., 2002). All members of this group were found to be evolutionary conserved with homologues found already in yeast. The second class contains the remaining two proteins, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis, which have evolved more recently (Jackson, 2002). All enzymes are ubiquitously expressed in all cell types and ligate double strand DNA breaks irrespective of sequence homology. They have also been found to generally repair double strand breaks thus representing repair function beyond gene rearrangement (Bassing et al., 2002).

During the DNA religation the coding and signal ends within the postcleavage synaptic complex are processed differently. The signal ends are joined precisely with each other in a head-to-head fashion thus forming a circular DNA fragment that is finally lost when the cell proliferates (Jung and Alt, 2004). However, the religation of the residual coding strands can proceed in a much more imprecise way which is contributing to further diversity to the joined region and is started by the opening of the hairpin loops. This cleavage often occurs asymmetrically resulting in a single-stranded tail containing a few nucleotides of the coding sequence followed by a stretch of nucleotides derived from the respective complementary DNA strand. DNA repair enzymes replenish the missing DNA strand by complementary nucleotide addition thus generating palindromic DNA regions. The newly introduced nucleotides are therefore termed P (palindromic) nucleotides (Lafaille et al., 1989; McCormack et al., 1989).

A second group of newly introduced nucleotides found in the joined regions are N nucleotides (non-templated). These N sequences are the result of the action of terminal deoxynucleotidyl transferase (TdT), an enzyme, which catalyzes an addition of nucleotides to the 3' ends of symmetrically cleaved hairpin loops of coding ends (Landau et al., 1987; for review see Benedict et al., 2000). N-nucleotide sequences at VD and DJ junctions can gain a length of up to 15-20 bp and are often G-rich due to preferential usage of dGTP by the enzyme (Max, 1999). They are mainly restricted to the heavy chain VDJ-junctions, because TdT expression ceases before the rearrangement of the immunoglobulin light chain genes. However, exceptions have been reported (Hiramatsu et al., 1995; Bentolila et al., 1999). The third mechanism generating diversity is rather the opposite of the two described so far. It concerns the loss of nucleotides of the J regions. This process known as 'nibbling' may be the result of exonucleases (Kenter and Tredup, 1991). The specific enzyme responsible for this process, has not been identified yet.

All of the three mechanisms presented here occur either alone or in combination, e.g. nibbling preceding N-nucleotide insertion during VDJ arrangement. Their more or less random nature gives rise to an enormous number of variant immunoglobulin chains, all displaying different antigen binding potential, distinct from the germline encoded sequences. However, these diversification mechanisms can also result in non-functional rearrangements as frame-shift mutations might be introduced. They lead to abortion of translation by encounter of stop-codons in the newly acquired reading-frames in the C-region and are therefore termed non-productive (non-functional) rearrangements.

1.4.4 Diversification by somatic hypermutation

The mechanisms contributing to diversification regarded so far take place during the formation of the B cell receptors in development and are essentially independent of antigen. However, even after the formation of BCR in mature cells its specificity can be altered. The underlying process is termed somatic hypermutation (summarized in Diaz and Casali, 2002). Its activation leads to the acquisition of point mutations within the variable regions of the heavy and light chain genes, thus, achieving further diversification. Although, these are distributed over the whole variable region of an antibody, distinct regions can be defined that display a high density of mutations, therefore known as hotspots. These are mostly found in the CDR regions.

The enzyme regarded to be the causative factor of this process is activation-induced cytidine deaminase (AID). The underlying mechanism is not completely understood yet, e.g. the question whether the target is RNA or DNA. This is due to the sequence homology of AID with the RNA-editing enzyme APOBEC-1 that is known to convert cytidine into uracil. However, several studies from the groups of Neuberger and Alt demonstrate direct interaction of AID with single stranded DNA (Lee et al., 2004; Besmer et al., 2004; Neuberger et al., 2005).
Somatic hypermutation is activated after a B cell recognizes an antigen via its receptor and requires T cell help. In some of these B cells the mutations will result in specificities that confer a stronger binding to the encountered antigen. This will allow the expansion of successful specificities, i.e. antibodies of higher affinities, a phenomenon also known as affinity maturation. It takes place in the germinal centers of the peripheral lymphoid organs (Franklin and Blanden, 2004).

Summarizing the diversification mechanisms described in this chapter: there are four main mechanisms for generation of diversity, that shape the BCR/antibody V-region. Starting with the existence of different V-, D- and J-region gene segments, combinatorial diversity is generated by the different combinations of these segments during assembly. This is further amplified by integration of additional P or N nucleotides or removal of coding sequences by ‘nibbling’. Additionally, the assembly of the antibody molecule itself provides further means for diversity - as the antigen binding sites are products of heavy and light chain V-region combinations. And finally, the diversification process is enhanced by the introduction of point mutations within V regions during the response to antigen.

1.4.5 Class switch recombination

BCRs and antibodies, respectively, can not only differ within their variable regions but also by their constant parts. B cells that after the completion of their development have not yet encountered antigen, are termed naïve B cells. These B cells express BCRs of two different classes, IgM and IgD, bearing the same variable region. The gene segment encoding the δ-chain lies directly 3’ of the μ-chain gene segment. The primary Ig transcript contains both C-regions in addition to the variable segments. Differential splicing gives rise to both BCRs (Max, 1999; Frazer and Capra, 1999).

During an immune response the IgM/IgD H-chain classes initially used can be substituted by the classes located further downstream in the locus. The underlying mechanism is called class switch recombination (CSR, see Chaudhuri and Alt, 2004). Class switch is started upon antigen-encounter and appropriate T cell help. It is mediated by special DNA sequences, so called switch regions, positioned in the intron between the rearranged VDJ region and the μ-gene as well as in the 5' regions preceding the respective downstream C-region gene segments except Cδ. CSR involves a recombination event between two S regions, with to a different downstream C_H gene (Figure 1.9). Interestingly the above mentioned enzyme AID is essential for CSR. Most likely it acts on the single stranded DNA that is generated during the activation of a particular C region for switching (Chaudhuri and Alt, 2004). As different antibody classes mediate different effector functions in concert with other cells or molecules of the immune system, the class switch mechanism provides the means for efficient clearance of a particular antigen i.e. a pathogen from the body.

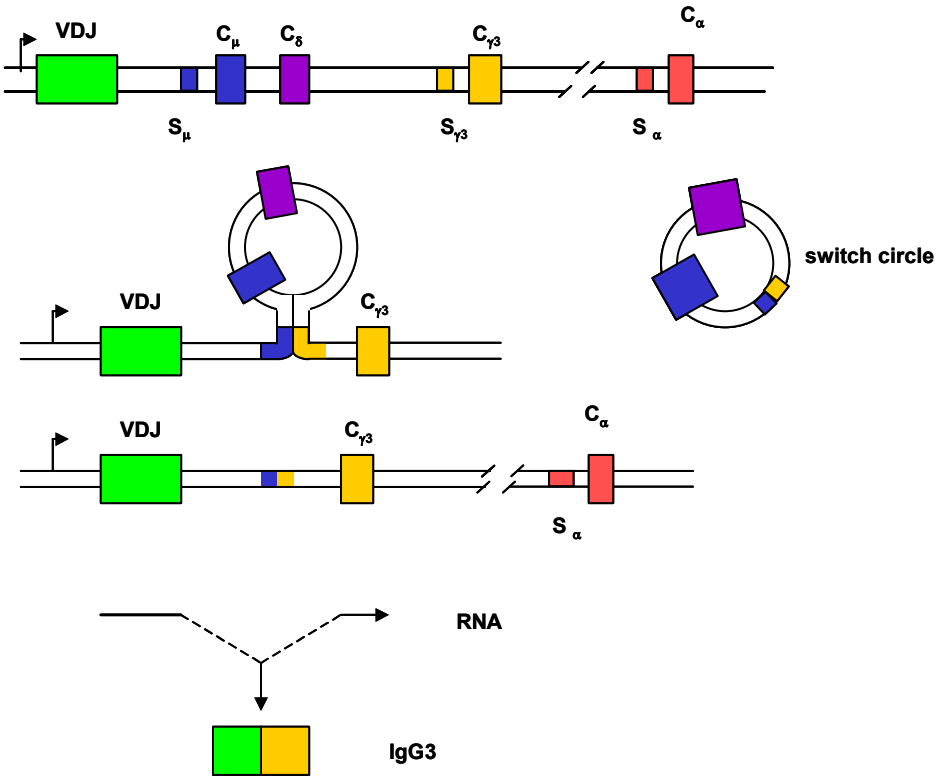


Figure 1.9. Schematic representation of the class switch mechanism. The episomal switch circle contains the intervening sequences removed during CSR. The new genomic order in this example may give rise to further switch events.

1.5 B cell development

All the processes described above can be assigned to particular stages in B cell development. These stages that are characteristic for particular steps during BCR assembly are paralleled by the differential expression of a collection of intracellular and surface marker proteins (Rolink et al., 1999).

1.5.1 Early events - B cell lineage commitment

B cell development is first observed in the fetal liver with early precursors found in the omentum and splanchnopleura. After birth B cell development takes place in the bone marrow (Herzenberg, 2000; Berland and Wortis, 2002). Starting point for the generation of B cells is the commitment of pluripotent, self-renewing hematopoietic stem cells (HSCs). HSCs are defined by their ability to give rise to all of the hematopoietic lineages *in vivo* and sustain the production of these cells for the life span of the individual. HSCs are discernable by their Thy-1^{lo}, Sca-1^{hi} (Ly6A/E), c-kit^{+} surface expression pattern. Moreover adult HSCs lack all the lineage markers (Lin^{-}) of mature cells from the hematopoietic system, i.e. CD3 (T cells), CD45R/B220 (B cells), Ly6G/Gr-1 (granulocytes), Ter119 (erythrocytes), Mac-1/CD11b (macrophages) (Wognum et al., 2003).

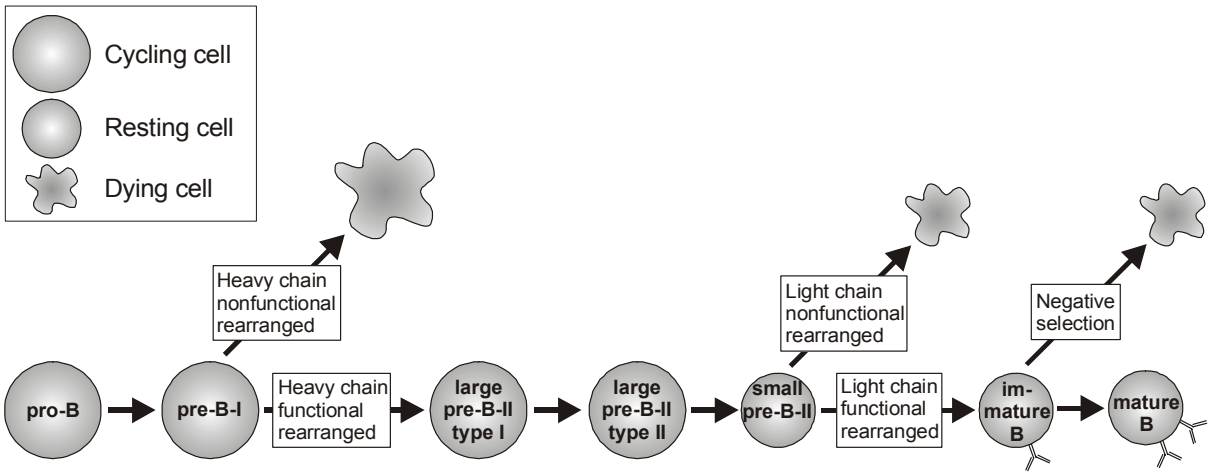
The first differentiation step results in the appearance of multipotent progenitor (MPP) types. This differentiation step is accompanied by the loss of long-term self-renewal characteristic for HSCs and the expression of the tyrosine kinase receptor Flt3, also known as Flk2 (Adolfsson et al., 2001; Christensen and Weissman, 2001). These early progenitor cells can on one hand give rise to an IL7 receptor negative ($IL7R^{-}$) common myeloid progenitor (CMP, Akashi et al., 2000) that has the potential to differentiate into all myeloid cell types (erythrocytes, megakaryocytes, granulocytes and macrophages). On the other hand, MPPs can differentiate into the earliest lymphoid progenitors (ELPs) which already initiate *Rag1* and *Rag2* expression and can undergo D_HJ_H rearrangement at the IgH chain locus (Igarashi et al., 2002). ELPs still can give rise to the $IL7R^{+}$ common lymphoid progenitor (CLP) in bone marrow and CLPs have residual potential to generate T, B, dendritic and natural killer (NK) cells (Kondo et al., 1997;Busslinger, 2004).

The entrance into the subsequent CLP-2 stage is marked by the onset CD45R/B220 expression. Cells at this stage also start to express the complement component C1q like receptor C1qRp, a widely used marker in analysis of B cell development (Norsworthy et al., 1999; Petrenko et al., 1999, see also Rolink et al., 2002). This transmembrane protein can be found on all succeeding stages of immature B cells but is lost on mature B cells.
Correlated with the induction of CD19, the cells finally enter the pro-B cell stage during which they complete the first part of the BCR receptor assembly: the rearrangement of the D_H and J_H genes (Hardy et al., 1991; Li et al., 1993; Li et al., 1996). The absence of mature B cells in $Rag^{-/-}$ mice has been traced to a block of B cell development at this stage (Mombaerts et al., 1992; Shinkai et al., 1992). Besides this main $B220^{+}CD19^{+}$ fraction a second $B220^{-}CD19^{+}$ fraction of cells was identified (Montecino-Rodriguez and Dorshkind, 2002). Interestingly, although expressing the CD19 molecule, which is usually associated with B cell lineage, these cells show nevertheless the potential for the generation of macrophages. However, these cells do not display the potential to generate T cells or NK cells.

1.5.2 B cell receptor formation

The pro-B cell stage mentioned above is the first in a series of seven defined stages that reflect different events during the assembly of the B cell receptor. Like the preceding ones these stages have been characterized and defined by means of differential surface marker expression. Additionally, the sequential expression of components of the BCR as well as a panel of intracellular molecules engaged in BCR formation characterize the single steps during the developmental progress.

Two nomenclatures are commonly used to define these stages in the bone marrow (Hardy et al., 1991; Rolink and Melchers, 1991). The nomenclature by Rolink and Melchers not only takes surface marker expression and the respective rearrangement states into account but also considers different cell sizes as indicators for proliferation (large cells) or rest (small cells). Based on this nomenclature the events during BCR formation will be described (Figure 1.10).



		pro-B	pre-B-I	large pre-B-II type I	large pre-B-II type II	small pre-B-II	immature B	mature B
Ig gene loci	**H:**	N.R.	DJ	VDJ	VDJ	VDJ	VDJ	VDJ
	L:	N.R.	N.R.	N.R.	N.R.	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$	$V_\kappa J_\kappa$ $V_\lambda J_\lambda$
Cell surface markers	**B220**	+	+	+	+	+	+	+
	CD19	+	+	+	+	+	+	+
	C1qRp	+	+	+	+	+	+	-
	c-kit	+	+	-	-	-	-	-
	IL-7R	+	+	+	+/-	-	-	-
	CD25	-	-	+	+	+	+/-	-
	CD40	-	-	-	-	+/-	+	+
	CD23	-	-	-	-	-	-	+
	sIgM	-	-	-	-	-	+	+
	sIgD	-	-	-	-	-	+/-	+
Intracellular markers	**Rag1**	-	+	-	+/-	+	+ (protein)	-
	Rag2	-	+	-	-	+	+ (protein)	-
	TdT	-	+	-	-	-	-	-
	$I\mu^0$	+	+	+	+	+	+	+
	SL	+	+	+	-	-	-	-
	cyto-μ	-	-	+	+	+	+	+
	κ^0	-	-	-	+	+	+	+
	κ/λ	-	-	-	-	-	+	+

Figure 1.10. Cellular stages of B cell development in the bone marrow. H: heavy chain locus. L: light chain locus. N.R.: not rearranged, +: detectable. protein: detectable only on protein level, SL: surrogate light chain. cytoμ: cytoplasmic μ chain, $I\mu^0$ germline transcripts of the heavy chain locus, κ^0, λ^0: germline transcripts of the respective light chain loci.

Initially, DJ rearrangement on both chromosomes takes place in the pro-B cell stage. The transition into the following pre-B-I cell stage is marked by the loss of Flt3 but the cells remain ckit$^+$IL7R$^+$. The recombinase machinery as well as TdT are expressed at the pre-B-I stage (Grawunder et al., 1995a).

Pre-B-I cells are often used in differentiation models as they can be cultivated and maintained in this stage *in vitro* in the presence of IL7 or IL3 and start to differentiate upon removal of the cytokines (Rolink et al., 1991; Winkler et al., 1995). Moreover, transferred into lymphocyte deficient mice they can give rise to fully mature B cells indicating their exclusive commitment to the B cell lineage.

The next step in heavy chain formation, the joining of V_H to DJ_H segments occurs at the transition from pre-B-I to the pre-B-II stage (Rolink et al., 1999). Whenever the V_HDJ_H rearrangement on one allele is productive and leads to a μ-heavy chain that potentially can pair with a light chain, a pre-B cell receptor (pre-BCR) is formed and exported to the cell membrane.

The pre-BCR

The pre-BCR has a structure that is reminiscent of the BCR (Figure 1.11). It consists of two newly formed μ-heavy chains associated with two so called surrogate light chain (SL) heterodimers that mimic a functional light chain (Hendriks and Middendorp, 2004). Surrogate light chains consist of two subunits, the VpreB and the λ5 protein, which are non-covalently associated. λ5 is encoded by a single gene and two isotypes exist for VpreB-proteins, the VpreB1 protein found to be expressed in all cells expressing the λ5 gene, the VpreB2 protein only found to be co-expressed in approximately 30% of such cells. However, both can contribute to a functional pre-BCR (Kudo and Melchers, 1987; Karasuyama et al., 1994; Dul et al., 1996). The VpreB and λ5 genes are located on chromosome 16 in mice closely linked to the λ locus (Gerdes and Wabl, 2002).

The pre-BCR is fully capable to initiate signaling mediated via its two accessory molecules Igα and Igβ. This step is crucial for further B cell development providing a first positive selection step as cells that fail to rearrange a functional heavy chain and thus cannot provide a functional pre-BCR complex undergo apoptosis. However, the trigger of the signaling events is still unclear. Two different scenarios are discussed. The first supports the idea that an extrinsic ligand stimulates the pre-BCR signaling. This is based on the finding that galectin-1, a stromal cell derived lectin, is required to

induce Igα-Igβ phosphorylation in a human pre-B cell line (Gauthier et al., 2002). Furthermore soluble murine or human pre-BCR molecules show specific binding to stromal-cell associated heparan sulphate, controlled by the non-Ig-like unique tail of λ5 (Bradl and Jack, 2001; Gauthier et al., 2002).

On the other hand, Ohnishi and Melchers showed that the non-Ig-like unique tail of λ5 mediates cell-autonomous pre-BCR signaling (Ohnishi and Melchers, 2003). They proposed that either the pre-BCR interacting with neighboring pre-BCRs serves intrinsically as its own ligand or that alternatively pre-B cells express a molecule on their cell surface that has binding sites for the non-Ig-like unique region of λ5. However, in all signaling scenarios λ5 mediates a crucial impact on signaling (Hendriks and Middendorp, 2004).

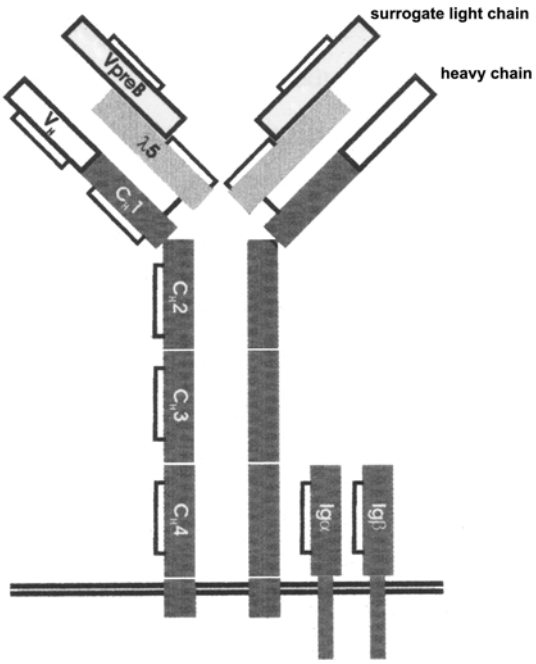


Figure 1.11. The pre-BCR. Two rearranged μ-chains form a complex with two surrogate light chains consisting of the two noncovalently associated chains VpreB and λ5. The two known VpreB proteins show homology to Vλ segments, the λ5 gene has homology to the Jλ and Cλ gene segments.

Signaling initiated through the pre-BCR induces clonal expansion of the $Igμ^+$ pre-B cells with IL7 playing a crucial role as pre-B-cell specific proliferation factor (Milne et al., 2004). As a result, pro-B cells differentiate to the next developmental stage of large, cycling pre-B cells. Recently, GL7 has been described as a novel marker

characterizing this stage (Murasawa et al., 2002). In cycling pre-B cells, TdT expression is shut down and the *Rag1* and *Rag2* genes are transiently downregulated upon signaling from the pre-BCR. Thus, the recombination machinery is turned off (Grawunder et al., 1995a). This happens as soon as one of the two heavy chain alleles is functionally rearranged which explains why every single B cell expresses only one type of heavy chain, a phenomenon termed allelic exclusion. This principle is also valid for rearranged light chain loci. The mechanism appears to be very stringent since only occasionally B cells are found that seem to have circumvented it (with a frequency of 1 in 10^4; Barreto and Cumano, 2000). The respective molecular events that finally lead to allelic exclusion, have still to be unraveled (Mostoslavsky et al., 2004).

After a limited number of cell divisions, during which surrogate light chain expression ceases, the large pre-B-II cells stop cycling and differentiate into small, resting pre-B-II cells. The transition is associated with reactivation of the rearrangement machinery as now the second step of receptor assembly occurs: the light chain rearrangements (Rolink et al., 1999). Germline transcripts, i.e. transcripts of unarranged Ig gene segments, that indicate an accessibility of the particular Ig loci for the recombination machinery are in the case of κ already present in the large pre-B-II cell stage (Grawunder et al., 1995b, Engel et al., 1999). In contrast germline transcripts for the λ-locus become detectable not until the small pre-B-II stage (Engel et al., 1999).

In large pre-B-II cells the rearrangement of κ gene segments is initiated and precedes in small pre-B-II cells (Grawunder et al., 1995b; Engel et al., 1999). If this results in a functional κ light chain it can bind to the μ chain and is expressed as IgM molecule on the cell surface together with Igα and Igβ. If the rearrangement of κ genes is non-productive, rearrangement at the λ L-chain loci takes place (Rolink et al., 1999). As soon as a successful light chain rearrangement is achieved the expression of the BCR on the cell surface results in the inhibition of additional rearrangements at all light chain loci. This leads to the expression of either a κ or a λ chain. As only one light chain isotype is thus expressed per B cell, the phenomenon is called isotype exclusion. In combination with allelic exclusion also found for the light chain genes only one species of light chain and thereby only a single type of

antibody per cell is produced. Collectively, these limitations are referred to as 'haplotype exclusion' (Nemazee et al., 2002).

Small pre-B-II cells that produce a functionally rearranged light chain differentiate into immature B cells (Rolink et al., 1999). These cells express a functional IgM BCR receptor on their surface that is associated with Igα and Igβ and are characterized as $IgM^{hi}IgD^{lo}C1qR^{+}$ bone marrow cells. Within the immature stage potential harmful anti-self specificities are cleared from the newly generated B cell pool. In contrast to previous selection steps that probed the functionality of the receptor complex, selection at the immature stage is designed to probe the receptor-ligand interaction, since now the BCR is for the first time able to interact with antigens. It has been estimated, that 40-60% of all surface IgM positive B cells in the bone marrow may have autoreactive specificities (Spanopoulou et al., 1994; Xu et al., 1998), making an effective selection in terms of self-protection indispensable. Three main mechanisms act at this stage in order to evade autoimmunity: receptor editing, apoptosis and anergy.

Editing

Although IgM expression induces light chain allelic exclusion, surface IgM expression does not necessarily terminate rearrangement. Instead, secondary rearrangements can occur, replacing the original specificity with a new, non-autoreactive one (Edry and Melamed, 2004). This process is called receptor editing and takes place mainly at light chain loci since the required RS configuration may still exist. However, receptor editing has also been observed at the H chain loci where a V in the VDJ unit is replaced by an upstream germline V_H segment (Reth et al., 1986; Kleinfield and Weigert, 1989).

Apoptosis

If receptor editing fails, escape from autoimmunity is usually assured by apoptosis of the cells with anti-self BCR specificities (Nemazee and Burki, 1989; Hartley et al., 1993; Sandel and Monroe, 1999). Therefore immature B cells from the bone marrow undergo apoptosis when stimulated with high levels of anti-Ig mimicking self-antigen encounter (Norvell et al., 1995). The process to eliminate self-reactive B cells by apoptosis is also known as clonal deletion and is mainly observed when a multivalent, often membrane-bound self-antigen is recognized.

Anergy

Cells that encounter soluble self-antigens during the immature stage might enter a state of anergy. Anergic B cells show downmodulation of IgM surface expression and impaired BCR signaling in response to antigen. They also fail to provide appropriate signaling for the activation of T helper cells. Due to these defects they are not activated to secret antibodies. In normal mice anergic B cells show furthermore a shorter half-life compared with mature B cells ensuring the rapid elimination of autoreactive specificities (summarized in Cornall et al., 1995).

1.5.3 Peripheral maturation finalizes B cell development

B cell development in the bone marrow is closely associated with stromal cells. During the transition to the immature state, however, the immature B cells become independent of stromal factors, migrate inside the central sinus and leave the bone marrow via the blood stream (Carsetti et al., 2004). They are then passively transported to the spleen, where they locate within the outer periarteriolar lymphoid sheat (PALS) (Figure 1.12). They still can be assigned to the immature B cell compartment due to their $IgM^{hi}IgD^{lo}CD21^{lo}CD23^{-}$ $CD24^{hi}$ surface markers and the expression of C1qRp. Such B cells are also referred to as transitional B cells because they are in transit from the bone marrow and represent a distinct short-lived developmental transitional stage from immature to mature B cells. At least two major subsets of transitional B cells exist, transitional type 1 (T1), which specifies the freshly entered cells from bone marrow and transitional type 2 (T2), that are at a $IgM^{hi}IgD^{hi}CD21^{hi}CD23^{hi}CD24^{hi}$ $C1qRp^{+}$ stage and are already located within the follicles (Su et al., 2004).

After 1-2 days T2 cells develop into $IgM^{+}IgD^{hi}CD21^{+}CD23^{+}CD24^{lo}$ follicular or $IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo}$ marginal zone B cells. This final maturation step involves the loss of the marker protein C1qRp that marks nascent and immature B cells (Rolink et al., 1999; Loder et al., 1999). Now the B cells have finished their maturation and are fully capable to exert their respective effector functions in response to antigen.

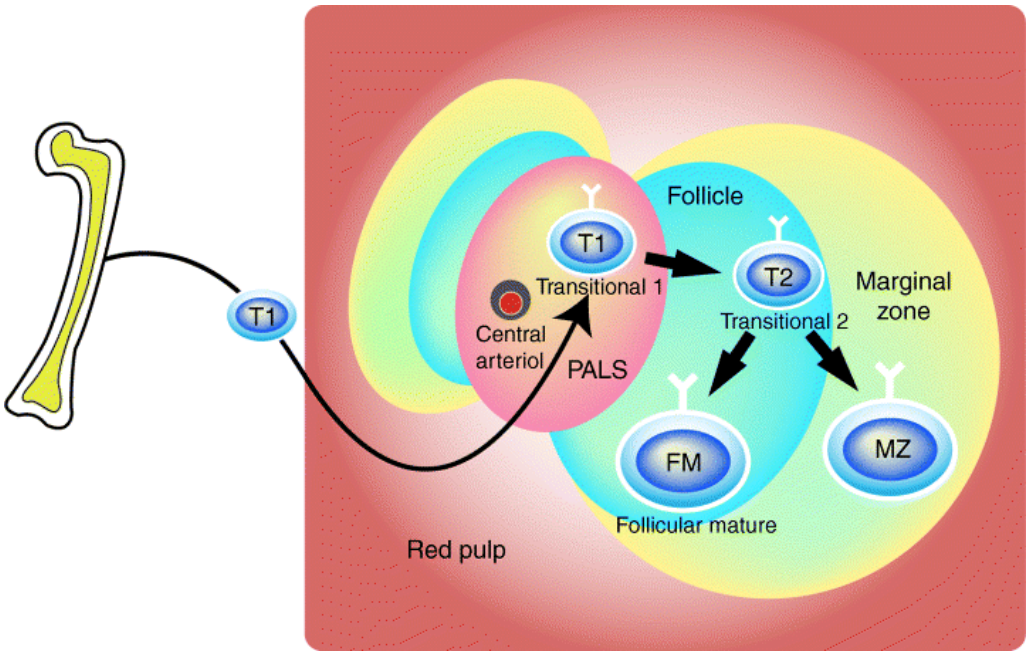


Figure 1.12. Peripheral B cell development. Immature B cells leave the bone marrow as short-lived, transitional B cells. T1 cells travel through the bloodstream and enter the spleen where they reside within the periarteriolar lymphoid sheath (PALS). T1 cells differentiate into T2 cells that reside within the splenic follicle until they differentiate into mature B cells (Su et al., 2004).

1.5.4 Fetal liver as a different site of B cell development

The developmental steps shown in the chapters before describe mechanisms mainly based on observations made on B cells generated in the adult bone marrow. However, B cell development is already initiated during embryonic stages, with the fetal liver (FL) as the main site of hematopoiesis. The overall concept of B lymphocyte development found in bone marrow holds true also for fetal liver with regard to expression of cell surface and intracellular molecules as well as the correlated sequential rearrangement of Ig loci. However, also differences can be found.

Concerning the B lymphocyte development in FL many progenitors have been identified that show a common myeloid/lymphoid potential. Thus a tripotent progenitor, which gives rise to T cells, B cells and macrophages has been described (Lacaud et al., 1998). Furthermore bi-potential precursors giving rise to B/myeloid progeny (Ohmura et al., 1999; Kawamoto et al., 2000) and a rare population of B/macrophage progenitors that have no potential to differentiate into other myeloid cells (Cumano et al., 1992) were found. Moreover a CLP population equivalent to the

CLP in BM with regard to cell marker expression was identified in FL. In contrast to BM CLP these cells are capable to generate also DCs and macrophages in addition to T, NK and B cells (Mebius et al., 2001). Interestingly bi-potential T/B progenitors which have been found in adult bone marrow (Kondo et al., 1997) have not been found in the fetal liver up to now. In summary, these observations indicate that FL lymphoid precursors retain the capacity to generate macrophages. In contrast lymphoid BM progenitors - with exception of the B/macrophage progenitor population mentioned earlier (1.5.1) - do not display this potential (Douagi et al., 2002).

Other differences between fetal liver and murine bone marrow B cell precursors concern the differential expression of particular molecules. Recently performed studies in our lab that compared $CD19^{+}ckit^{+}$ cells from FL and bone marrow of BALB/c mice in microarray based experiments revealed a total number of 88 genes (70 known and 18 expressed sequence tags (EST)) differentially expressed between both sites (Düber, 2004). They also confirmed the previous made observation that pre-B cells generated in the fetal liver e.g. lack MHC class II expression whereas in the BM this molecule is already expressed on pre-B cell stage (Hayakawa et al., 1994; Lam and Stall, 1994). Another example is the gene for a regulatory myosin light chain (precursor lymphocyte-specific regulatory light chain or PLRLC) (Oltz et al., 1992) that is expressed in BM pre-B cells but cannot be found in fetal liver pre-B cells. However, the most prominent example of a differentially expressed molecule is that of TdT. In contrast to B cell precursors from BM FL precursors express little or no TdT during VDJ rearrangement of the heavy chain loci (Li et al., 1993). Accordingly B cells from fetal liver contain no or only few N nucleotides in the VDJ junctions of their heavy chains in contrast to B cells generated in adult BM (Feeney, 1990; Li et al., 1993; Gilfillan et al., 1993). Thus the lack of N nucleotide diversification at the heavy chain junctions is usually taken as an indicator for fetal origin of a B cell.

Another difference between fetal and adult B cell development was revealed by the analysis of the effect of pre-BCR assembly/signaling on the proliferation of pre-B cells in stromal culture. Although the expression of a heavy chain transgene (capable of associating well with the SL chains of the pre-BCR) resulted in proliferation of adult bone marrow pre-B cells, the same chain inhibited proliferation of pre-B cells derived from fetal liver (Wasserman et al., 1998, see also Hardy et al., 2000). In contrast, a heavy chain transgene that failed to assemble efficiently with the SL chain had less suppressive effect on fetal proliferation. Therefore Wasserman et al. proposed that

pre-BCR signaling is important to promote B cell development in bone marrow but induces exit from cell cycle in fetal liver derived pre-B cells which are the main source for B-1 cells.

1.6 B-1 cells

The identification of B-1 cells as additional population besides B-2 cells some 30 years ago was followed by many observations that addressed the different nature of both populations. Upon growing data, the question after the origin of both mature subsets became a controversial issue resulting in two hypotheses to explain their occurrence. The linchpin concerning both consist in the question if B-1 and B-2 cells originate from one or two different lineages.

1.6.1 Origin of B-1 cells

The initial hypothesis concerning B-1 cell development came from Herzenberg and coworkers (reviewed in Herzenberg, 2000). It was based on cell transfer studies in which fetal liver cells transferred into irradiated mice could reconstitute both, B-1 and B-2 cell populations, whereas transferred adult bone marrow only led to the generation of B-2 cells. Therefore, they proposed a model that suggested development from different, committed precursors, hence, B-1 and B-2 cells being descendants of two different lineages. Later, the fetal omentum and paraaortic splanchnopleura were shown to give rise exclusively to B-1 cells (Solvason et al., 1991; Godin et al., 1993). In addition, when all B cells were depleted in mice after birth the newly formed B cells showed a fast recovery of B-2 cells in contrast to an impaired generation of B-1 cells (Lalor et al., 1989a; Lalor et al., 1989b; Hamilton and Kearney, 1994). This was taken as further indication that B-1 and B-2 cells derive from different lineages with their progenitors present at particular sites and time periods during ontogeny.

In contrast, the second hypothesis suggests an ‘induced differentiation’ for B-1 cell generation (Cong et al., 1991; Haughton et al., 1993; Clarke and Arnold, 1998). It proposes that the B cell fate is determined by the nature and quality of the signaling mediated via its BCR. Based on their specificity, cells are thus selected either into the B-1 or B-2 compartment. Since the BCR is randomly assembled this hypothesis predicts one common progenitor for both B cell populations. The signaling events

upon (self) antigen encounter by slightly autoreactive or polyreactive specificities, possibly in addition of inappropriate T cell help, would select the cells into the B-1 compartment (Berland and Wortis, 2002).

Several observations have led to this proposal: *in vitro* experiments on splenic B-2 cells manipulating BCR signaling by anti-Ig treatment resulted in the conversion from splenic B-2 cells into cells with B-1 characteristic in terms of marker expression and responses to mitogenic stimuli (Rothstein et al., 1991; Cong et al., 1991; Wortis et al., 1995; Murasawa et al., 2002). Furthermore the fetal/neonatal repertoire is skewed towards the expression of immunoglobulins that bind TI-2 antigens (as for example found in the microbial flora of the gut). The adult repertoire generates these specificities rarely (e.g. due to N nucleotide insertion upon TdT expression). Thus, only few B-1 cells originate from adult bone marrow.

Table 1.1. Extract of genetic alterations that affect B-1 cell numbers (Berland and Wortis, 2002).

Alterations that decrease B-1 cell numbers	References
Mutations of positive regulators of BCR signaling	
Btk$^{-/-}$ or point mutation of btk	Khan et al., 1995; Hendriks et al., 1996
PKC$\beta^{-/-}$	Leitges et al., 1996
PLC$\gamma^{-/-}$	Hashimoto et al., 2000; Wang et al., 2000
Deletion of P85α of PI-3 kinase	Fruman et al., 1999; Suzuki et al., 1999
CD19$^{-/-}$	Engel et al., 1995; Rickert et al., 1995
SLP65$^{-/-}$	Jumaa et al., 1999; Pappu et al., 1999
CD21/35$^{-/-}$	Ahearn et al., 1996; but see Molina et al., 1996
Vav$^{-/-}$	Tarakhovsky et al., 1995; Doody et al., 2001; Tedford et al., 2001
Mutation of B cell transcription factors	
Oct2$^{-/-}$	Humbert and Corcoran, 1997
Aiolos$^{-/-}$	Wang et al., 1998
NFATc$^{-/-}$	Berland and Wortis, 2002
Alterations that increase B-1 cell numbers	
Mutations of negative regulators of BCR signaling	
SHP-1$^{-/-}$	Schmidt et al., 1998
CD22$^{-/-}$	O'Keefe et al., 1996; Sato et al., 1996a ; but see Otipoby et al., 1996; Nitschke et al., 1997
Lyn$^{-/-}$	Chan et al., 1997
CD72$^{-/-}$	Pan et al., 1999
Overexpression of positive regulators of BCR signaling	
CD19 transgenic	Sato et al., 1996b

In addition, many transgenic and gene targeted mouse models seem to provide evidence for the signaling based model. Mutations that weaken BCR signaling by alteration or deletion of positive regulators in the signaling pathways lead to decreased B-1 cell numbers. In contrast diminished or absent function of negative regulators leads to increased B-1 cell numbers (Table 1.1).

Moreover transgenic mouse models expressing a B-1 specificity develop mainly B cells belonging to the B-1 compartment, most of them expressing CD5. Conversely, in mice carrying B-2 derived transgenes, transgene expressing B cells belong almost exclusively to the B-2 compartment (Table 1.2).

However, supporters of the two-lineage model nevertheless argue, that the phenotypes observed in these transgenic mice could as well be attributed to different selection processes that B1 and B2 progenitors have to undergo (Herzenberg, 2000).

Table 1.2. Effect of Ig-transgene expression on B cell development (Berland and Wortis, 2002)

Transgene	Specificity	Source	Phenotype[a]	References
3-83	H-2K^{k}D^k	adult spleen	B-2	Nemazee and Burki, 1989
HyHEL-10	Hen Egg Lysozyme	adult spleen	B-2	Goodnow et al., 1988
V_H12/ $V_\kappa4$	Phosphatidyl choline (PtC)	CD5+ Lymphoma	B-1a	Arnold et al., 1994
V_H11/ $V_\kappa9$	Phosphatidyl choline (PtC)	B-1 cell	B-1a	Chumley et al., 2000
SM6C10	Thy-1	B-1 cell	B-1a	Hayakawa et al., 1999; Hayakawa et al., 2003
4C8	Mouse red blood cells	NZB spleen	B-1	Okamoto et al., 1992
2-12H	sM snRNP	MRL/lpr mouse	B-1a	Qian et al., 2001

[a] Predominant phenotype of B cells expressing the transgene

1.6.2 Features

B-1 cells are not defined by a single specific marker. In flow cytometry they can be distinguished from other B cells by differential expression using several cell surface markers. B-1 cells are IgM^{hi}IgD^{lo}CD23$^-$CD43$^+$B220^{lo} and constitutively express the IL5 receptor (Wetzel, 1989; Berland and Wortis, 2002). This is in contrast to B-2 cells that are IgM^{lo}IgD^{hi}CD23$^+$CD43$^-$B220$^+$. In addition, peritoneal B-1 cells are classified into CD5$^+$ B-1a and CD5$^-$ B-1b sister populations.

In contrast to the generally small resting follicular B-2 cells, B-1 cells are large granular cells and exhibit more side scatter in flow cytometry (Berland and Wortis,

2002). Furthermore, B-1 and B-2 cells differ also physiologically. Peritoneal B-1 cells have been shown to respond readily to phorbol esters without additional stimuli whereas cell cycle entry of B-2 cells requires ionomycin in addition to phorbol esters (Rothstein and Kolber, 1988b). Moreover, B-1 cells fail to enter cell cycle in response to anti-Ig stimulation and undergo apoptosis whereas the same stimulus leads to proliferation of B-2 cells (Rothstein and Kolber, 1988a; Morris and Rothstein, 1993; Bikah et al., 1996). According to their constitutive expression of IL5R, peritoneal B-1 cells elicit stronger proliferation upon IL5 stimulation then splenic B-2 cells (Wetzel, 1989; Baumgarth et al., 2005).

Several studies showed differences between B-1 cell populations of the peritoneum and the spleen. The longest known example is the differential expression of Mac-1 (CD11b/CD18) between peritoneal and splenic B-1 cells, the former expressing the molecule whereas the latter lacking it (Kantor et al., 1992). Moreover, CCR2 and CCR3 transcripts have been found in splenic but not in peritoneal B-1a cells (Rothstein et al., 2000). Splenic B-1 cells furthermore express much less IL-10 mRNA than peritoneal B-1 cells and peritoneal B-1 cells express constitutively phosphotyrosinylated STAT3 which localizes in their nuclei whereas splenic B-1 cells do not (Fischer et al., 2001). Furthermore splenic B-1 cells have recently been reported to express the Notch family members Notch-1 and Notch-2 and other Notch-related genes at significantly higher levels then peritoneal B-1 cells (Tumang et al., 2004). Another difference between peritoneal and splenic B-1 cells concerns a more functional aspect. In contrast to peritoneal B-1 cells splenic B-1 cells fail to respond upon phorbol ester stimulation (Fischer et al., 2001).

1.6.3 Function

B-1 cells have been shown to be the major source of natural serum IgM (Herzenberg et al., 1986; Lalor et al., 1989a; Baumgarth et al., 1999; Baumgarth et al., 2005). These antibodies are produced in the absence of exogenous antigenic stimulation and provide a first line of defense against the infection by pathogens (Ochsenbein et al., 1999; Baumgarth et al., 2000). Accordingly, many antibody specificities against discrete microbial or viral components can be found in the natural antibody repertoire (Baumgarth et al., 2005). Furthermore many of these antibodies are poly- and weakly autoreactive and bind specific antigen with low-affinity (Baumgarth et al., 2005). Several studies characterized these particular features of B-1 cell derived antibodies

and provided evidence for the importance of B-1 specificities in the defense against pathogens.

Antibodies of the B-1 cell derived T15 idiotype that consist of $V_H1/V_\kappa22$ family member associations, have been shown to play an important role in protection from infection by *Streptococcus pneumoniae* (Briles et al., 1981; Yother et al., 1982). Furthermore antibodies with this idiotype can bind to oxidized low-density lipoprotein (LDL), which may contribute to its clearance from blood thus suggesting a role in prevention of atherosclerosis (Shaw et al., 2000). Similarly, such antibodies could have an impact during the course of this vascular disease as they may bind to atherosclerotic plaques and initiate an inflammatory response.

Other prominent specificities found in 5-15% of peritoneal B-1 cells are those for phosphatidyl choline (PtC) (Mercolino et al., 1988; Wang and Clarke, 2004a). PtC is a glycolipid component of common plasma membranes and is exposed on senescent erythrocytes and on many other cell types that have been treated proteolytically (Kawaguchi, 1988). Thus anti-PtC specificities are examples for the many autoreactive specificities found in the B-1 cell pool (see 1.6.6). Anti-PtC antibodies in normal mice are encoded by either $V_H11/V_\kappa9$ or $V_H12/V_\kappa4/5H$ heavy light chain combinations and show restrictions in the heavy and light chain CDR3 regions (Wang and Clarke, 2004a). The PtC specificity of $V_H11/V_\kappa9$ and $V_H12/V_\kappa4/5H$ antibodies was revealed in studies that investigated their binding to mouse red blood cells (MRBCs) after treatment with the proteolytic enzyme bromelain (Br) (Kawaguchi, 1988). Therefore, they are also termed anti-BrMRBC antibodies. Besides their potential to bind senescent cells, anti-BrMRBC have been also shown to recognize thrombin-treated mouse platelets (Kawaguchi, 1989). Therefore, it has been proposed that these antibodies may overtake a kind of physiological “housekeeping” function by removal of senescent cells (Hardy et al., 2004).

Another prominent B-1 cell derived specificity is directed against LPS (Su et al., 1991). Almost all natural antibodies that react against this bacterial cell wall component can be traced back to B-1 origin. These antibodies are important for the clearing of LPS. This was indicated by studies that used antibody deficient mice. They showed increased susceptibility to death after LPS injection but the phenotype could be rescued by administration of normal mouse serum containing natural antibodies prior to LPS injection (Reid et al., 1997).

Natural antibodies have also been shown to control early stages of the immune reaction against influenza viruses (Baumgarth et al., 2000). In the absence of natural IgM mice infected with influenza virus showed markedly decreased survival and an increased viral replication in the lung (Baumgarth et al., 2000). B-1 cells were identified as the source for the virus binding antibody whereas B-2 cells contributed only minimally to the anti-viral IgM antibodies. However, seven days after the infection with virus increased titers of B-2 derived IgM were observed whereas the titer of natural anti-virus IgM stayed constant. Thus B-1 cells do not respond to the infection with increased IgM production (Baumgarth et al., 2000). However, they provided a stable titer of IgM keeping the infection in check until the adaptive immune response mediated by B-2 cells could take place.

1.6.4 B-1 cell contribution to immune responses

It is possible to distinguish antigens upon the requirements of the elicited immune response. Responses to some antigens require the presence of mature T cells, therefore classified as T cell dependent (TD) antigens whereas others do not and are therefore called T cell independent (TI) antigens. The T cell independent antigens moreover can be further subdivided into TI-1 and TI-2 antigens. TI-1 antigens, often also referred as innate antigens, are polyclonal activators of B cells leading to their differentiation into antibody-secreting cells irrespective of their specificity. Antigens that belong to this class are LPS, peptidoglycans, lipoproteins. However, bacterial cell walls also contain TI-2 antigens which are typically repetitive polymers such as capsular polysaccharides from bacteria. They lack an intrinsic polyclonal B cell-activating property. Prominent members of this second class are pneumococcal cell walls and dextrans but also synthetic haptenized derivatives such as TNP-Ficoll or NP-Ficoll (summarized in DeFranco, 1999).

B-1 cells have been shown to participate in TI-2 responses (Claflin et al., 1974; Forster and Rajewsky, 1987; Martin et al., 2001). Transfers of B-1 cells into allotype-congenic mice revealed their in vivo responsiveness towards the TI-2 antigen α-1-3 dextran (Forster and Rajewsky, 1987). Furthermore, the above mentioned T15 idiotype has been shown not only to contribute to natural serum IgM but also to the response during *S. pneumoniae* infection (Claflin et al., 1974; Masmoudi et al., 1990; Martin et al., 2001). The T15 antibodies were produced in addition to the already

existing idiotypes in the sera. Infection of mice devoid of T cells demonstrated the T cell independency of this process (Martin et al., 2001).
However, some TI-2 antigens like TNP-Ficoll or NP-Ficoll do not lead to a B-1 cell driven TI-2 immune response which is instead provided by the second group of TI-2 responsive cells - the marginal zone B cells (MZ) of the spleen (Hayakawa et al., 1984; Berland and Wortis, 2002). Because B-1 and MZ B cells are positively selected, indicated by their skewed repertoire, and can respond rapidly to pathogens they are believed to provide a “natural memory” to the immune system.

1.6.5 Natural memory

The term ‘natural memory’ has been developed in analogy to the term immunological memory which reflects the fact that e.g. B cells that have once responded to a certain antigen can in case of a second encounter with the antigen respond faster and more efficiently. Thus memory B cells provide already appropriate antibody classes and an affinity matured repertoire (Mc Heyzer-Williams L.J., 2005). The immunological memory therefore reflects adapted skills for every individual gained during the course of its life.
In contrast the ‘natural memory’ is suggested as a phenomenon adapted during evolution. The repertoire and reactivity pattern of natural antibodies and B-1 and MZ BCRs, respectively, is remarkably stable within each species and moreover even between species. This is in part explained by the frequent usage of germline V-genes that lack N nucleotide insertions. However, it is argued as well that the stability also reflects the innate usage of a series of V genes that by evolutionary pressures have been selected in the genome to provide an inherent legacy of specificities suitable for protection against pathogens (Martin and Kearney, 2001; Baumgarth et al., 2005).

1.6.6 Autoreactivity

In contrast to B-2 cells a large fraction of B-1 cells express self-reactive antibodies (Baumgarth et al., 2005). The anti-BrMRBC/PtC antibodies mentioned above are one example for such a B-1 cell derived autoreactive specificity (see 1.6.3). Other autoreactivities are directed against the cell membrane component phosphorylcholine (PC) (Masmoudi et al., 1990), immunoglobulins (rheumatoid factor) (Casali et al., 1987), carbohydrate epitopes, single stranded DNA (Casali et

al., 1987) and cell surface molecules such as Thy-1 (CD90), an antigen expressed on most thymocytes and peripheral T cells (Schlesinger, 1965; Martin and Martin, 1975; Hayakawa et al., 1999).

In general, low affinity antibodies are not pathogenic in normal animals. Only when these self- and polyreactive antibodies undergo affinity maturation and isotype switching and thus become high-affinity IgG antibodies, they might become pathogenic. Although somatic mutations are usually not seen in peritoneal B-1a cells (Forster et al., 1988; Berland and Wortis, 2002), it could be demonstrated that B-1 cells have the potential to undergo somatic hypermutation as well as class switch (Taki et al., 1992). Thus B-1 cells might have the potential to contribute to pathological situations (Rothstein, 2002; Viau and Zouali, 2005). Accordingly, there are a number of autoimmune diseases in mouse and human where an association between disease and B-1 cells is discussed. Among these are the autoimmune hemolytic anemia (AHA) observed in NZB mice as a result of anti-red blood cell antibody production (Howie and Helyer, 1968) and murine lupus erythematosus (SLE) observed in F_1 hybrids of NZB and NZW mice (Hayakawa et al., 1983). In addition, a B-1 cell contribution has been suggested in rheumatoid arthritis (Youinou et al., 1990) and Sjogren's syndrome (Dauphinee et al., 1988). All of these diseases are associated with elevated numbers of $CD5^+$ B cells

To provide a pool of autoreactive specificities, seems on the first glance more risky than beneficial. However, the observation that monoclonal antibodies reactive with self antigens also react with pathogens sheds a new light on this situation (Mercolino et al., 1986; Kearney et al., 1987; Claflin and Berry, 1988; Pecquet et al., 1992). The assumption is that the natural antibody repertoire may indeed be composed entirely of autoantibodies that also react with pathogens (Baumgarth et al., 2005). The pool of such reactivities thus would provide immediate immune protection that controls an infection until the adaptive immune system can fill in. This explanation is moreover perfectly in line with the observation that B-1 cells are positively selected by autoantigen (Hayakawa et al., 1999).

1.6.7 Positive selection of B-1 cells by autoantigen

The important impact of autoantigen upon B-1 cell selection has been shown by work from Hayakawa and colleagues using an anti-thymocyte autoantibody (ATA), a self-

specificity found amongst $CD5^+$ B cells. This antibody is encoded by V_H3609 and Vκ21C germline genes and binds to a carbohydrate epitope on the Thy-1/CD90 glycoprotein. The expression of the V_H3609µ chain as a transgene in mice resulted in the generation of both B-1 and B-2 cells. However, the frequency of $CD5^+$ B cells was increased. Moreover, the B-1 cells produced high titers of serum ATA - generated through pairing of the transgene with endogenous Vκ21C light chains. In contrast, B-2 cells in the spleen expressing the same heavy chain were found to be associated with a diverse repertoire of light chains and did not contribute to the high serum ATA levels. In the absence of the autoantigen (i.e. in Thy-1 deficient mice) no ATA-expressing B-1 cells were detected and in accordance no high ATA serum titer was observed. Thus both the accumulation of ATA $CD5^+$ B cells and elevated level of serum ATA were dependent on the presence of the autoantigen (Hayakawa et al., 1999; Hardy and Hayakawa, 2004).

1.7 The L2 mouse model

Although an extensive amount of knowledge about B-1 cells has been accumulated since their discovery, many important aspects are still poorly understood. This is in part due to the low numbers of such cells found in normal mice. To avoid such problems in the present work, a transgenic mouse strain (L2) was used that has been extensively characterized before (Engel et al., 1998; Kretschmer et al., 2002; Kretschmer et al., 2003a; Kretschmer et al., 2003b; Kretschmer et al., 2004). These mice express a transgenic λ light chain obtained from the plasmacytoma MOPC315 ($\lambda 2^{315}$). Their major lymphoid organs contain almost exclusively B-1 cells while the development of conventional B-2 cells is inhibited in the bone marrow (Engel et al., 1998). In the spleen of L2 mice 5-10% $CD5^+$ B-1 cells are found which makes these mice extremely useful for the investigation of such cells. The only additional splenic B cell population are MZ B cells (Kretschmer et al., 2003a).

Studies carried out on the antibody repertoire of B-1 cells in L2 mice revealed a striking phenotype (Kretschmer et al., 2002; Kretschmer et al., 2003b). Since the heavy chain loci in L2 mice could theoretically rearrange randomly during B cell development they could give rise to a very heterogeneous pool of different µ-chain specificities. However, the sequencing of transcribed heavy chains from the B-1 cell pool of L2 mice showed pronounced oligoclonality. In addition some sequences could

be found repeatedly by single cell RT-PCR and dominated the repertoire. These particular specificities could be shown to be derived from independent cells that had undergone also independent rearrangements of their receptor genes (Kretschmer et al., 2002; Kretschmer et al., 2003b).

These dominant sequences could repeatedly be even found at the same frequency in the peritoneum of different individual mice. Thus, a substantial fraction of the B-1a cells expresses the same BCR on their surface providing an excellent molecular marker for these B-1 cells. The most dominant sequence was found in 15% of the peritoneal B-1a cells, a second in 13% and a third in 7,4 %. These data suggest a strong positive selection of such specificities probably by autoantigen (Kretschmer et al., 2002; Kretschmer et al., 2003b).

Only some of these specificities could be found at a reasonable frequency in the spleen. This suggested that there is little exchange between these two anatomical compartments. This was confirmed by migration studies in L2 mice. When peritoneal cells were transferred into the peritoneum of L2 mice very few transferred B-1 cells could be recovered from the spleen (Kretschmer et al., 2003b). In accordance, gene chip analysis revealed significant differences between these two cell populations (Kretschmer et al., 2003b).

Subsequent work furthermore demonstrated an impact of the spleen on the maintenance of the dominant clones. The dominant specificities found in the peritoneal cavity were lost after splenectomy. This, suggests a dependency of peritoneal B-1 subpopulations on a splenic survival factor, which in accordance with the previous observations was speculated to be a splenic autoantigen (Kretschmer et al., 2004).

1.8 Aims of this work

The results of the gene chip analysis on B-1a cells from spleen and peritoneal cavity of L2 mice could be explained in two ways. Splenic and peritoneal B-1 cells are descendants of two different cell lineages or the microenvironment imprints a particular gene expression pattern onto B-1 cells that arise from the same precursors. To decide this question diagnostic genes should be defined for the two populations from the gene chip analysis. Their expression should then be analyzed in cells that were recovered after transfer of peritoneal cells from spleen and

peritoneum of lymphopenic mice in which transferred peritoneal B-1 cell are known to home to the spleen and the peritoneum. Along these experiments novel, so far unknown, markers should be defined for B-1 cell by using the gene chip results.
The dominant specificities could be regarded as unique B-1 cell specific markers. Therefore, antibodies with these specificities should be further analyzed. To this end hybridomas had to be established to obtain monoclonal antibodies representing these specificities. Using such antibodies, first anti-idiotypic antibodies should be generated that would greatly simplify the analysis of the dominating specificities. Furthermore, these B-1 cell derived antibodies should be used to search for the antigen that is recognized by them and it should be investigated how they behaved *in vivo* since they were supposed to be directed against an autoantigen.

2 Material and methods

2.1 Mice

The L2 mouse line (IgH^a) has been described previously. (Engel et al., 1998). Transgenic and non-transgenic mice were used at the age of 6-15 week old mice. Transgenic mice were crossed to BALB/c background for more than 20 generations and kept as heterozygotes. L2 mice of the IgH^b allotype were obtained crossing L2 mice to CB20 mice that are congenic with BALB/c mice but carry the IgH^b haplotype. To obtain mice with the B cell specific deletion of the VCAM-1 gene mice homozygous for the loxP flanked (floxed) VCAM-1 gene (Terry et al., 1997) were crossed with CD19cre mice (Rickert et al., 1995). $Rag1^{-/-}$ mice on BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, USA). $Ig\alpha^{-/-}$ mice on BALB/c background were obtained from Michael Reth (Freiburg) (Pelanda et al., 2002).

2.2 Flow cytometry and cell sorting

Single cell suspensions were obtained by flushing lymphoid organs or the peritoneum of mice with ice-cold IMDM (Iscove's modified Dulbecco's medium, GibcoBRL/Invitrogen). Erythrocytes in splenic, thymic or bone marrow preparations were lysed by incubation with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 2-5 min on ice.

Blocking: $5x10^5$-10^6 cells per 100 µl were incubated in FACS-buffer (2% FCS and 2mM EDTA in PBS (8 g NaCl; 0.2 g KCl, 1.44 g Na_2HPO_4 x $2H_2O$, 0.2 g KH_2PO_4 in 1 l H_2O; pH 7.0)) containing Fc-block (clone 2.4G2, protein G purified from concentrated hybridoma supernatants or obtained from Pharmingen), mouse serum and/or Endobulin (human IgG, Baxter). The blocking was carried out on ice for 15-20 min.

Staining: Cells were stained for 10-15 minutes on ice using antibodies coupled to FITC, PE, APC or Biotin from the list below. Samples were washed twice with FACS-buffer afterwards. Biotinylated antibodies were counterstained in a second

step with streptavidin-APC (Pharmingen) or streptavidin-R-PE conjugates (Southern Biotechnology Associates) for 10 min on ice followed by a washing step with FACS-buffer. Flow cytometry was carried out using a FACSCalibur® (Becton Dickinson). Cell sorting was carried out on a MOFLO (Cytomation), or a FACSVantage® (Becton Dickinson). Data were analyzed using Summit® v3.1 software (Cytomation) or CellQuest software (Becton Dickinson).

Antibodies

Monoclonal antibodies	
IgM^a (DS-1)	Pharmingen
IgM^b (AF6-78)	Pharmingen
IgD^a (AMS9.1)	Pharmingen
IgD^b (217-170)	Pharmingen
CD5 (53-7.3)	Pharmingen
VCAM-1 (429(MVCAM.A))	Pharmingen
CD19 (1D3)	Pharmingen or protein G purified from concentrated hybridoma supernatants*
CD3 (500A2)	Pharmingen or protein G purified from concentrated hybridoma supernatants*
CD23 (B3B4)	Pharmingen
CD21(7G6)	Pharmingen
Polyclonal antibodies	
goat anti-mouse JAM-1 (Lot #: GJO01)	R&D Systems

*Antibodies obtained from hybridoma supernatants were conjugated to FITC (FLUOS, Roche Diagnostics) or Biotin (Biotinamidohexanoic acid N-hydroxysuccinimide ester, Sigma) according to standard protocols .

8H10 stainings were performed using supernatants from 8H10 hybridoma (rat-anti mouse L2P3, isotype IgG2b) culture and revealed by an mouse anti-rat IgG (H+L) Cy5-conjugated $F(ab')_2$ fragment (Jackson ImmunoResearch). L2P3, L2M15 and MOPC104E stainings were detected using the IgM^a-FITC coupled antibody (Pharmingen) as secondary reagent.

2.3 Transfers

Cells obtained from peritoneal lavages of L2 mice were washed twice with PBS prior to transfer.

Intravenous transfer: $1.6x10^7$ cells per mouse were transferred into the tail vein of $Rag1^{-/-}$ mice. After 2.5 weeks mice were sacrificed and cells from spleen and peritoneum were prepared. Cells from both compartments of individual mice were analyzed in flow cytometry using antibodies specific for IgM^a, CD5 and VCAM-1. Residual cells from both organs were pooled, stained with anti-CD5 and anti-IgM^a antibodies and sorted to obtain samples for RT-PCR.

Intraperitoneal transfer kinetics: $7.8x10^6$ cells per mouse were transferred i.p. into $Rag1^{-/-}$ mice. Splenic and peritoneal cell preparations of transferred mice were analyzed after 7, 15, 23, 29 and 32 days by flow cytometry using antibodies specific for IgM^a and CD5 according to standard protocols. Spleen cells were additionally stained with anti-VCAM-1 antibody.

Intraperitoneal transfer: $2,6x10^7$ cells were transferred into the peritoneum of $Rag1^{-/-}$ mice. After 3 weeks cells from peritoneum and spleen of these mice were prepared and cells from both compartments of individual mice were analyzed in flow cytometry. To this end, cells were stained with anti-IgM^a, anti-CD5 and anti-VCAM-1 antibodies. Residual cells from both compartments were pooled, stained with anti-CD5 and anti-IgM^a antibodies and sorted.

Transfer of VCAM-1^-CD19^+ peritoneal cells: Cells from peritoneal lavages of L2 mice were stained with anti-VCAM-1 and anti-CD19 antibodies and sorted. VCAM-1^- $CD19^+$ cells were mixed with cells obtained from peritoneal lavages of $Ig\alpha^{-/-}$ mice at a ratio of 2:1. From this mixture, $7.2x10^6$ cells per mouse were transferred into the peritoneal cavity of $Rag1^{-/-}$ mice. After two weeks mice were sacrificed and spleen and peritoneal cavity cells were analyzed by flow cytometry using antibodies against IgM^a, CD5 and VCAM-1.

2.4 Preparation of RNA

Total RNA from sorted cells or hybridomas was prepared with TRIzol® Reagent (GIBCO BRL®) according to the manufacturer's protocol. After preparation, RNA was solved in 10 µl DEPC treated water (Ambion) and transcribed into cDNA. Alternatively, possible DNA contamination in total RNA was eliminated prior to cDNA generation, by DNase1 treatment (DNA-free^{TM}, Ambion) according to the supplied protocol. Briefly, extracted RNA was incubated with DNase1 (2 units) at 37°C and after 30 min treated with DNase1 Inactivator. Samples were spun down at 10.000x g for 1 min and supernatants collected.
In case of sorted cells, photometrical determination of RNA prior to cDNA synthesis was not possible because of low cell numbers available. For these samples the amount of cDNA was estimated by PCR with primers for Hprt.

2.5 cDNA synthesis

To transcribe cDNA, 3-5 µl RNA in DEPC treated water, 1 µl oligo-d$(T)_{12-18}$ (500 µg/ml, Amersham Biosciences), 0.5 µl RNAsin (40 U/µl, Promega) were adjusted with RNAse free water to a total volume of 12 µl per sample (Mix1) and incubated for 10 min at 70 °C. Samples were cooled on ice for two minutes and reaction mix containing 4 µl 5x RT buffer (Invitrogen), 2 µl 0.1 M DTT, 1µl 10 mM dNTP and 1µl Superscript II RNaseH^- (200 U/µl, Invitrogen) was added. To control for genomic DNA contaminations additional samples were included in which the enzyme was substituted with water. Samples were incubated for 60 min at 42°C and 2 min at 95°C thereafter. cDNA was stored at -20°C.

2.6 PCR

Oligonucleotides: Primer for PCR (except VCAM A-D primers) were selected by DNASTAR, Primer Select software (Lasergene) based on sequences obtained from the Ensembl Genome Browser (www.ensembl.org). All primer pairs, except Ccr3 and Fpr1, were designed as intron spanning primer pairs to control for genomic DNA contamination. Ccr3 and Fpr1 PCRs were conducted including control samples in which Superscript II enzyme was omitted during cDNA preparation. Primers for

VCAM A-D were selected according to Araki et al., 1993. All primers were purchased from Invitrogen.

Primer	5’ 3’
Adamdec1 for	CATACAGCTGGAAGGGACAAGAC
Adamdec1 rev	ACTGAGAAGCTGGGCGTGGTTAT
Adml for	AGAACACAACTGGCCCCCTACAA
Adml rev	CACCCGCACCTATATCCTAAAGAG
Api6 for	GACTGCAACGGAACGGAAGACACG
Api6 rev	CCAAACGACTCAAAAGGCAAGACC
Ccr3 for	CTCGCTATCCAGAAGGTGAAGAAG
Ccr3 rev	GTGGAAAAAGAGCCGAAGGTGTT
Cd5 for	AGAAAAAGCAGCGTCAGTGGATTG
Cd5 rev	TCAGGATGGAGGGGATGC
F11r for	ACCCGGAAGGACAATGGAGAGTAT
F11r rev	TGGGCCTGGCAGTAGTATTCAC
Fpr1 for	GAACTTGGCCATTGCTGACTTTTG
Fpr1 rev	GACGGCCACCTTCCTCTTCTCTAC
Hpgd for	GCAGGCGTGAACAATGAGAAAAAC
Hpgd rev	CACAAAGCCTGGGCAAATGACA
Lpl for	CGAGAGGATCCGAGTGAAAG
Lpl rev	CTAATGCTGGAAGACCTGCTATG
Mrc2 for	TTTTACGAGAAGTTGGGGTCAGG
Mrc2 rev	TTTGGTCAGCTTTGGTTGTAATGG
Spic for	CAGAGGCAACGCTAACTACTATGG
Spic rev	CAGCTTTCTCCGGATTTTTATGAT
Vcam1 (=Vcam7/8) for	TCCCTAATGTGTATCCCTTTGACC
Vcam1 (=Vcam7/8) rev	AGGCTGCAGTTCCCCATTATTTAG
VCAM A for	CAACGATCTCTGTACATCCC
VCAM B rev	AGAGGCTGTACACTCTGCCT
VCAM C for	AAGGATCCGGTACCAAGCAGAGACTTGAAATGCC
VCAM D rev	CCCTTGAACAGATCAATCTCC
Wee1 for	TTTGCTCTTGCTCTCACAGTCGTA
Wee1 rev	CCATTTGGGCTTTCTTGAGTTCT

PCR: PCR reactions were conducted in a reaction volume of 20 µl per sample using the HotStarTaq® DNA polymerase kit from Quiagen.

Reaction mix:

1.2 µl	primer mix (containing 10 pmol/µl forward and 10 pmol/µl reverse primer)
2.0 µl	10x PCR buffer containing 15 mM $MgCl_2$ (Quiagen)
1.6 µl	2.5 mM nucleotide mix (Bioline)
0.2 µl	HotStarTaq DNA polymerase (5U/µl; Quiagen)
13.6 µl	Ampuwa® water (Fresenius Kabi)

19 µl in each tube

1µl cDNA was used as template in each PCR reaction. For PCR reactions polymerase was activated at 95°C for 15 min. Optimized conditions for each primer pair were used (20 s 94°C, 40 s 50-60°C, and 20 s 72°C, final extension 72°C 10 min). Depending on the redundancy of each cDNA, 30-40 cycles were performed.

Fifteen microliters were analyzed in gel electrophoresis.

Threefold serial dilutions of the cDNA were used for semiquantitative RT-PCR and normalized according to expression of the housekeeping gene Hprt.

Reaction tubes: 0.2 ml Micro-Strips & Caps (Abgene® House)

2.7 Gel electrophoresis

PCR products or restricted DNA were analyzed in agarose gel electrophoresis on 0.8 % or 2% gels, respectively (SeaKeM® LE Agarose, Cambrex) in TBE-buffer (90 mM Tris-Borate, 2 mM EDTA, 0.11% (v/v) acetic acid). Ethidium bromide was added to the gels to reveal DNA bands under UV light. The size of DNA fragments was determined by comparison with DNA fragments of known size.

DNA-markers: SmartLadder SF, 100 bp- 1kb (Eurogenetec); peQ gold 1 kb DNA-Leiter (PeqLab) (used for gel electrophoresis during Southern experiments).

Documentation was performed using the E.A.S.Y Win32 gel documentation system and software (Herolab).

2.8 Southern blotting

$CD19^+$ or $CD3^+$ cells from flox/flox; cre/+ mice were sorted according to standard protocols. Yield for $CD3^+$ and $CD19^+$ splenic cells were between 4-5x10^6 cells. The pooled peritoneal fraction yielded 6.4x10^5 $CD19^+$ cells.

Preparation of genomic DNA: For the preparation of genomic DNA, 720 µl tail buffer (100 mM Tris HCl (pH 8.5), 5 mM EDTA (pH 8.0), 200 mM NaCl, 0.2% SDS) and 30 µl proteinase K (Sigma) of a 10 mg/ml stock solution in H_2O was added to the cell samples and shaken over night at 54°C. The next day, samples were spun down at room temperature (10 min, 20000 x g) and supernatants were transferred into a new tube containing 600µl 2-propanol. Tubes were gently inverted to promote DNA precipitation. Then samples were spun down for 5 min at 10000 x g. Supernatants were removed, the pellets washed with 500 µl 70% ethanol and subsequently dried at room temperature. Pellets were resuspended in 50 µl TE 10/1 (10 mM Tris (pH 7.6), 1mM EDTA) and incubated overnight in a thermomixer at 54°C to properly dissolve the DNA.

DNA digestion and blotting procedure: For Southern blot analysis, DNA was digested overnight at 37°C with BamHI (New England BioLabs) in a 60 µl reaction volume containing BamHI restriction buffer (New England BioLabs), 1 mM spermidine (Sigma), 1 mM DTT (Sigma), 100 µg/ml BSA (New England BioLabs), 50 µg/ml Rnase A (Serva), and 15U BamHI per reaction. Subsequently, the total volume of each sample was loaded to an agarose gel (0.8%) and separated by electrophoresis. DNA was then transferred to a nylon membrane (Gene Screen plus, NEN^{TM}Life Science Products) by capillary force under alkaline conditions. After the transfer, the membrane was washed with excess 2x SSC for 1-2 minutes (20x SSC: 3 M NaCl, 0.3 M Tri-sodiumcitrate, pH 7.2). Finally, the DNA was fixed to the membrane by drying it for 2 h at 80°C.

*Labelling of the probes: Bgl*II/*Spe*I restriction fragments used as probes for Vcam1 were as described by Terry et al., 1997 and kindly provided by Dr. Angela Schippers, EI, GBF. The probe was labeled by incorporation of [α-^{32}P] dCTP (Amersham Biosciences) using the "Ladderman^{TM}Labelling Kit" (TaKaRa) according to the manufacturer's protocol. Non-incorporated nucleotides were removed using ProbeQuant^{TM} G-50 Micro Columns (Amersham Biosciences). After addition of 35 µl sonicated salmon sperm DNA (10 mg/ml) the probe was heated at 95°C for 5 min and was used for the hybridization of the blot.

Hybridization and detection: To wet the membrane and to remove excess salt, the blot membrane was briefly dipped into deionized water before prehybridization. Then it was prehybridized in a rotating hybridization oven for 45 min at 65 °C using QuickHyb® Hybridization Solution (Stratagene). Subsequently the labeled and denatured probe was added to the solution and hybridized for one hour.
After hybridization the following washing steps were carried out:

1) two times shortly with 2x SSC/0.1% (w/v) SDS at room temperature
2) two times for 15 min with 0.1x SSC/0.1% (w/v) SDS at 58°C

Afterwards the membrane was wrapped in plastic wrap. Autoradiography was carried out using a BIOMAX MS film (Kodak) and an intensifying screen at -80 °C. Exposure was performed over night and developed using the Curix 60 automatic developer machine (Agfa).

2.9 Generation of hybridomas

Pooled cells from either spleen or peritoneum of two L2 mice were sorted for expression of CD5 and IgM (purities: spleen 91%, peritoneum 93%) and stimulated *in vitro* for 48h with 20 µg/ml LPS (Bacto Difco) in OptiMEM medium (GibcoBRL) containing 5% heat inactivated FCS (Integro), 100 µg/ml penicillin/streptomycin (Biochrom AG), 100 µg/ml gentamycin (Biochrom AG) and 250 µM ß-mercaptoethanol (Serva). Cells were then fused to non secreting P3X63Ag8.653 mouse myeloma cells (Kearney et al., 1979).
Cell fusion: For fusion, myeloma cells and activated lymphocytes were washed with serum-free IMDM (GibcoBRL) and mixed at ratio of myeloma:lymphocyte of 20:1. The mixture was spun down (5 min, 180 x g) and the supernatants discarded. After

loosening the pellet, the cells were incubated for 5 min at 37°C using a water bath. While gently shaking the tubes in the water bath, 500 µl polyethyleneglycole solution (PEG 1500; 50% (w/v) in 75 mM HEPES buffer (Boehringer)) were slowly added to the pellet over 60 seconds and tubes were agitated for an additional minute. 9 ml serum-free IMDM (GibcoBRL) were then added dropwise (3 ml/30 sec) followed by an additional incubation at 37°C (water bath) for 5 min. Cells were spun down (5 min, 180 x g) and the pellet was resuspended in HAz medium followed by an 30 min incubation at 37°C in the incubator. Hybridomas were then plated under limiting dilutions in HAz medium and only wells bearing single colonies were expanded for further analysis.

HAz-Medium: 0,5 mg Azaserine/6,8 mg Hypoxanthin (Hybri-Max, Sigma) were dissolved in OptiMEM medium (GibcoBRL) containing 5% heat inactivated FCS (Integro), 100 µg/ml penicillin/streptamycin (Biochrom AG), 100 µg/ml gentamycin (Biochrom AG) and 250 µM ß-mercaptoethanol (Serva).

Culture conditions: Hybridomas were cultivated at 37°C and 5.0% CO_2 in a humidified atmosphere.

2.10 Amplification and sequencing of IgM chain transcripts from hybridomas

Total RNA was prepared from 1-3 x 10^6 cells of the hybridomas. Reverse transcription and PCR were carried out sequentially in the same tube with primers for the amplification of the IgM V region ($V_H$cons: 5'-GAGGTGCAGCTGCAGGAGTCTGG-3'; C_μ1: 5'-ATGGCCACCAGATTCTTATCAGA-3') using the QIAGEN® One Step RT-PCR Kit according to the manufacturer's protocols. Reverse transcription was performed at 50°C for 30 min in 25 µl reaction mix. PCR conditions were 95°C 30s, 50°C 30s, 72°C 30s using 30 cycles. The PCR was completed by a final extension step for 10 min at 72°C.

PCR products were run on 2% agarose gels and extracted using QIAquick gel extraction kit (Qiagen). Purified products were directly sequenced. For sequencing the C_μ1 primer described above was employed. The sequencing reaction was carried out in a 11 µl reaction volume using the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit (PE Biosystems) applying the following programme: 96°C for 60 s,

25 cycles at 96°C for 60s, 55°C for 15s and 60°C for 4 min. After the reaction the DNA was precipitated. Briefly, 100 µl 2-propanol were added to the samples and mixed. After 15 min incubation at room temperature, the precipitate was spun down (45 min, 1420 x g, room temperature). The supernatant was removed by a subsequent centrifugation with inverted open tubes (1 min, 180 x g). Residual 2-propanol was evaporated on a thermocycler by incubation of the open tubes at 65°C for 2-5 min. Samples were stored at -20 °C until loading on the sequence gel and analyzed with an automatic DNA sequencer (ABI PRISM 377, PE Applied Biosystems) in the Department of Genome Analysis at the GBF.

Sequences were evaluated with the Sequencer^{TM} 3.1.1 program (Gene Codes Corporation). To this end they were aligned with the sequences of the four functional J_H gene segments to determine the J-usage. The V_H gene segment family was determined submitting the sequences to database search (Advanced BLAST Search and IgBLAST, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). The longest D element matches were used to assign the D element. At least five nucleotides had to match to assign a D segment. Nucleotides neighboring nucleotides at the ends of J, D or V gene segments and forming palindromic sequences with the neighboring nucleotides were assigned as P nucleotides, the remaining nucleotides were considered as N nucleotides.

2.11 Purification of IgM antibodies from cell culture supernatants

L2P3, L2M15, 2D4 (Young, Jr. et al., 1979) hybridoma cells and MOPC104E plasmacytoma cells were cultivated in IMDM (GibcoBRL/Invitrogen), 3% protein G adsorbed heat inactivated FCS (Integro), 100 µg/ml penicillin/streptamycin (Biochrom AG), and 250 µM ß-mercaptoethanol (Serva). Supernatants were collected from roller cultures and concentrated using an Amicon device (Series 8000, Millipore) with a 50K ultrafiltration membrane (Pall). After centrifugation of concentrated supernatants at 1620 x g (30 min) a pre-precipitation step was carried out. To this end a saturated ammonium sulfate solution (4.1M in H_2O) was added slowly and dropwise to the gently stirring supernatant at 4°C until 20% saturation was reached. Samples were then left stirring over night. As mouse antibodies start to precipitate at a 45-50% saturation this step was used as a pre-clearence step

removing debris that precipitates at lower ammonium sulfate concentrations. The solution was spun down at 1620 x g for 30 min and supernatants containing IgM molecules were submitted to a second over night precipitation adding saturated ammonium sulfate solution until a 45% saturation was reached. The solution was spun down thereafter at 1620 x g for 30 min and the pellet was suspended in PBS. After dialysis (Dialysis tubing Visking, exclusion limit MWCO 14 000, Roth) against PBS at 4°C an additional dialysis step against binding buffer from ImmunoPure® kit (Pierce) for the purification of IgM was carried out. IgM was purified using an immobilized Mannan-binding protein column according to the kit instructions thereafter. After purification IgM was dialyzed against PBS and stored at -80°C.

2.12 Generation of the 8H10 anti-idiotype antibody

Generation of the anti-idiotype antibody in rats and subsequent ELISA screenings for the identification of L2P3 binding hybridoma supernatants were kindly carried out in the lab of Dr. Elisabeth Kremmer at the GSF in Munich. Purified L2P3 IgM and L2M15 as well as MOPC104E used for ELISA screenings were provided from IgM preparations described above.

2.13 Analysis of IgM sequences of sorted IgM^+ $8H10^+$ cells

Peritoneal cells of six L2 mice were pooled and sorted for IgM and 8H10 expression. Total RNA was isolated from these cells using TRIzol® Reagent (GibcoBRL/Invitrogen). Reverse transcription and amplification of the resultant cDNA was done in one step according to the QIAGEN® One Step RT-PCR Kit instructions (Qiagen). For the amplication of IgM V region specific cDNA, the $V_H$cons (5'-GAGGTGCAGCTGCAGGAGTCTGG-3') and C_μ1 (5'-ATGGCCACCAGATTCTTATCAGA-3') primers were used (program: 50°C for 30 min followed by 30 cycles of 95°C 30s, 50°C 30s, 72°C 30s and a final extension step of 10 min at 72°C). The sample obtained from PCR was run on a 2% agarose gel and the PCR products were cut out and purified using the QIAquick gel extraction kit (Qiagen). PCR products were then cloned using the "TOPO TA Cloning kit" (Invitrogen). Plasmid clones were isolated using the GFX™ Micro Plasmid Prep Kit (Amersham Pharmacia Biotech) and the insertion of the cDNA fragments was tested by EcoRI restriction digestion of plasmid DNA aliquots prior to sequencing. Plasmid

DNA was sequenced using the $V_H$cons primer and sequences were evaluated as described in 2.10.

2.14 Determination of IgM clearence

10 µg of L2P3, L2M15, MOPC104E or 2D4 antibodies, respectively in PBS were injected intravenously into the tail vain of CB20/L2 mice. Groups of 3 mice were used except for the injection of L2M15, where only two mice formed one group. Mice were bled after 4 h, 1 day, 3 days, 5 days and 7 days and sera were prepared. Sera obtained before injection of the antibodies were used as controls for the ELISA (pre-bleed).

Sera: To obtain sera from mice, blood derived from the tail vein of mice was placed on ice for 2 hours to allow clotting of the blood. The clot was removed with a wooden toothpick and samples were spun down at 4 °C with 2700 x g for 10 min. Supernatants were transferred into new tubes and stored at -20 °C until usage.

ELISA: The kinetic of the clearence of injected IgM into CB20/L2 mice was determined with an allotype specific ELISA using an antibody against IgM^a that detects the injected IgM molecules and does not bind to IgM^b molecules of the CB20/L2 mice.

Coating buffer:	0.1M Na_2CO_3, 0.1M $NaHCO_3$; pH 9.6 in H_2O
Washing buffer:	0.1% Tween 20 in PBS with 5% FCS
Blocking buffer:	3% BSA in washing buffer
Dilution buffer:	1% BSA in washing buffer
OPD substrate:	1 mg/ml o-Phenylendiamin in Substrate Buffer; 0.3% H_2O_2
Substrate buffer:	0.2 M NaH_2PO_4 , 0.1 M Na_3-citrate; pH 5.0

Plates (MaxiSorb™ Immunoplates, Nunc) were coated over night at 4 °C with 2 µg/ml rat anti-mouse IgM antibody (clone II/41, Pharmingen) diluted in coating buffer. After washing, wells were blocked with 200 µl blocking buffer for two hours at RT and plates flicked to discard the fluid. The sera from different time points were diluted 1:100 in blocking buffer and added to the plate for a 2 h incubation at RT. After washing, 0.5 µ/ml biotinylated anti-mouse IgM^a antibody (DS-1, Pharmingen) in

dilution buffer was added and incubated for 1 h at room temperature. Plates were washed and horseradish peroxidase (HRP) conjugated streptavidin (Pharmingen) in dilution buffer was dispensed into the wells. Plates were incubated for 45 min at room temperature and washed. Then the ELISA was developed at RT for 25 min in the dark adding o-Phenylendiamin (OPD) as substrate for the peroxidase. Reaction was stopped adding 1 M H_2SO_4. Results were read using an ELISA-reader (BioRad 3550-UV microplate reader) at a wavelength of 490 nm.

Determination of IgM half-life: For each time point the mean value at OD490 was calculated from the data of individual mice of one group representing the IgM^a fraction in sera. The mean value at 4 h was arbitrarily set to 100% and the remaining mean values were correlated to it. Using Microsoft® Excel 2000, these data points were plotted in a semi-logarithmic diagram depicting the percentage of the IgM^a fraction versus time t, where $t = 0$ corresponds to the first data point at 4 h. To obtain the curve of the monophasic 2D4 clearance a trendline was determined by using a least-squares fit of the functional form $A \exp(-bt)$. A is the IgM^a percentage at $t = 0$ constrained to equal 100% and b is the elimination rate constant. Thus the half-life $T_{1/2}$ of IgM^a equated $\ln(2)/b$.

Each curve of the biphasic L2P3, L2M15 and MOPC104E kinetics is represented by the two-phase functional form $C \exp(-dt) + D \exp(-et)$. C and D are the extrapolated IgM^a percentages of the first (fast) and the second (slow) phase at $t = 0$, and d and e are the corresponding elimination rate constants ($d > e$). Using the built-in interpolation of Microsoft® Excel 2000 the approximated runs of these curves were obtained and plotted in a semi-logarithmic diagram. In order to determine the half-lives of the two phases, first, an extrapolated trendline was generated for the second phase of each curve applying the least-squares fit of the functional form $D \exp(-et)$. Subtracting data values of this trendline from values of the interpolated biphasic curve yielded data points that were used to determine the trendline representing the first phase. This trendline was generated using the least-squares fit of the functional form $C \exp(-dt)$, where $C + D$ was constrained to equal 100%. The half-lives $T_{1/2}$ for both the first and the second phase thus equated $\ln(2)/d$ and $\ln(2)/e$, respectively.

3 Results

B-1 cells have been extensively characterized. Nevertheless, it is often difficult to detect such cells in particular tissues or follow their migration. Thus the panel of available reagents and markers needs to be increased - an issue that was followed up in this work.

The background of the first part of this work was provided by microarrays that had been carried out before. Gene expression profiles from B cell populations located at different anatomical sites like peritoneal B-1a and B-1b cells as well as splenic B-1a cells from L2 mice had been obtained. Similarly, follicular B-2 cells as well as peritoneal and splenic B-1a cells from non-transgenic littermates had been analyzed (Kretschmer et al., 2003b). Based on those array data, genes had been classified into groups and two of them were further investigated during this work:

1) Genes that were expressed in splenic B-1a cells but revealed only background expression levels in peritoneal B-1a cells (Group 1),
2) Genes that were expressed in peritoneal and splenic B-1 cells but were absent in follicular cells (Group 2).

3.1 Group 1 genes

First genes expressed in splenic B-1a cells but not in peritoneal B-1a cells of L2 mice were analyzed. Differences between B-1 cells from spleen and peritoneum had been described before which include phenotypic, biochemical as well as functional aspects (Rothstein, 2002). These differences have led to the suggestion that B-1 cells from the two different anatomical sites may in fact represent two populations with different origin. On the other hand, they might simply reflect differences in B-1 cell function at the respective anatomical sites. Therefore, this question was tackled on the basis of the following assumptions: If peritoneal B-1 cells and splenic B-1 cells differ in their origin, a different developmental program should determine their phenotype. Hence, peritoneal B-1 cells brought into the splenic environment should fail to express specific genes found in B-1 cells of splenic origin. However, genes that are expressed due to functional requirements or environmental influences should also become expressed by peritoneal derived B-1 cells when residing in the spleen.

In order to investigate this the following experimental system was used: Peritoneal lavage cells obtained from L2 mice were transferred into lymphocyte deficient $Rag1^{-/-}$ mice. In these mice migration of intraperitoneally transferred B-1a cells from peritoneal lavages into the spleen had been shown before (Kretschmer et al., 2003b). Thus such transferred B-1 cells should be reisolated from spleen and peritoneum and the changes in expression of marker genes should be analyzed by RT-PCR and flow cytometry.

Microarray data were screened for genes expressed in splenic but absent in peritoneal B-1a cells (Figure 3.1). Then B-1a cells from spleen and peritoneum of female L2 mice were sorted to high purity (>98.5%) based on the markers $IgM^{hi}CD5^{+}$ and submitted to RT-PCR to confirm the data obtained by expression arrays.

All of the chosen genes displayed in Figure 3.1A showed the expected expression pattern by RT-PCR, i.e. being expressed in the splenic B-1a cells and showing low or no expression in the peritoneal B-1a cells.

To extend this analysis and to base the experiments on more quantitative data splenic and peritoneal B-1a cells were sorted again to high purity. Cells from male and female L2 mice were pooled to increase the number of cells analyzed. Semiquantitative analyses performed on the sorted L2 B-1a cells of two independent sorts confirmed the expression pattern found for Vcam1 and Spic (Figure 3.2). Furthermore, mRNA for an additional gene, Adamdec1, was also found in splenic B-1a cells but not detected in peritoneal B-1a cells. The data for Hpgd varied between the two independent cell sorting experiments performed. In the first experiment there was also some expression detected in peritoneal B-1a cells (data not shown). However, no expression was detected in peritoneal B-1a cells in the second sorting experiment (Figure 3.2). Expression patterns for Lpl and Mrc2 differed from the results displayed in Figure 3.1. Message from these genes was now also detected in peritoneal cavity B-1a cells and expression of Lpl in splenic B-1a cells was low in contrast to the preceding RT-PCR experiments. Similarly, expression of Fpr1, an additional gene analyzed, differed from the expectations based on array data, since it was found in B-1a cells of the peritoneal cavity.

A

L2		B-1a				B-1b	
Gene	**Affy_id**	**SP**	*SD*	**PEC**	*SD*	**PEC**	*SD*
Ccr3 (CCR3)	99412_at	**569**	90	**61**	0	**61**	0
Vcam1 (VCAM-1)	92559_at	**380**	67	**61**	0	**61**	0
Vcam1 (VCAM-1)	92558_at	**16760**	1398	**61**	0	**61**	0
Adamdec1 (Decysin)	98976_at	**314**	84	**61**	0	**61**	0
Spic (Spi-C)	103454_at	**752**	86	**61**	0	**61**	0
Lpl (lipoprotein lipase (LPL))	95611_at	**2104**	400	**69**	12	**482**	210
Hpgd (NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase)	93351_at	**1117**	93	**61**	0	**61**	0
Mrc2 (mannose receptor, C type (CD206))	103226_at	**2326**	191	**67**	8	**463**	55
Fpr1 (N-formyl peptide receptor)	99387_at	**869**	125	**63**	4	**61**	0

B

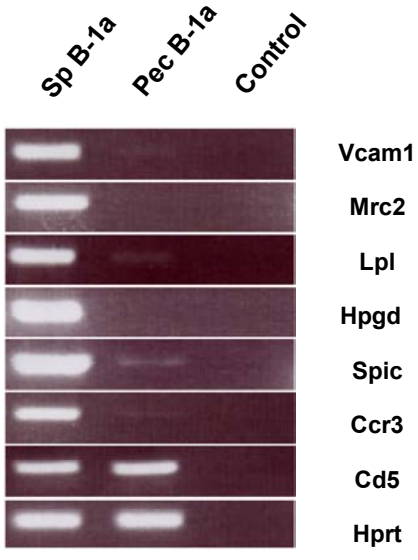
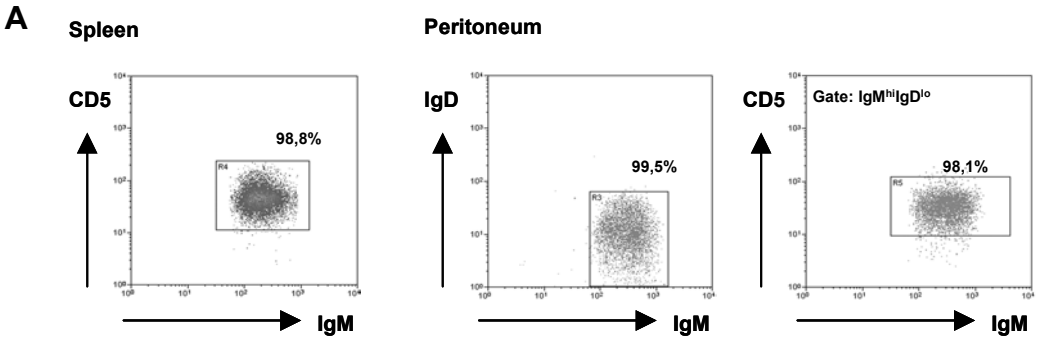


Figure 3.1. Differentially expressed genes among splenic and peritoneal B-1a cells and peritoneal B-1b cells from L2 mice. A) Array data of differentially expressed genes from spleen (SP) and peritoneum (PEC) as well as B-1b from peritoneum of L2 mice. The unique Affymetrix probe set identifier (Affy_id), mean average difference values are presented in bold letters and standard deviation (SD) of three replicate experiments are shown. Background values are represented by a mean average value of 61. B) Splenic B-1a (Sp B-1a) and peritoneal B-1a (Pec B-1a) were sorted according to expression of $IgM^{hi}CD5^+$ and analyzed by RT-PCR. Intron spanning primers were used to exclude genomic DNA. Control is water control.



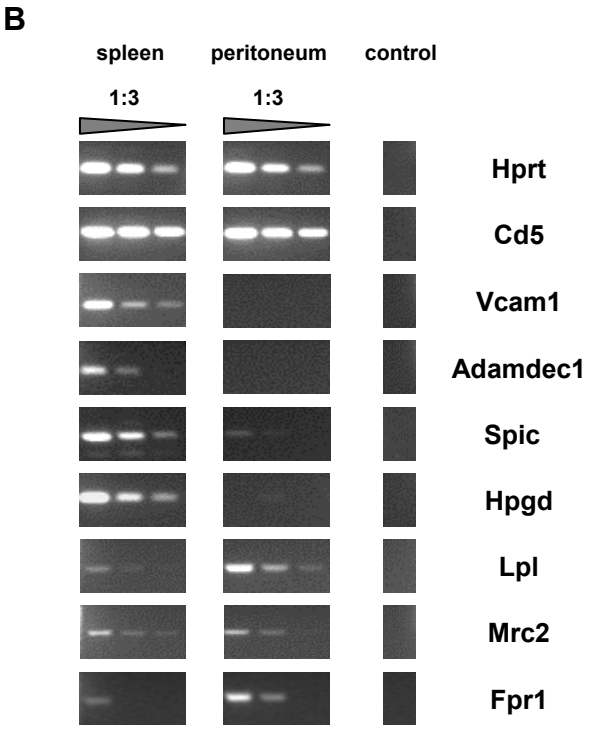
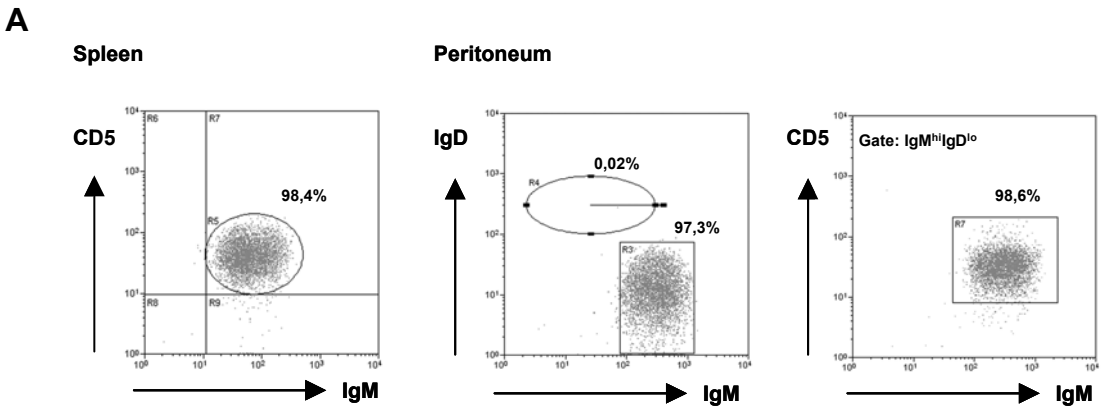


Figure 3.2. Semiquantitative RT-PCR analysis of sorted B-1a cells from peritoneum and spleen of L2 mice. A) Reanalysis of cells sorted from respective sites of L2 mice demonstrated a purity of 98,1% for peritoneal $IgM^{hi}IgD^{lo}CD5^{+}$ cells and 98,8% for splenic $IgM^{+}CD5^{+}$ cells. B) Semiquantitative RT-PCR analysis on sorted cells from A. cDNA was diluted threefold for each step. Data are representative for one of two experiments.

The expression of the respective genes was also tested in the B-1a cell population of non-transgenic mice. To this end, B-1a cells from spleen and peritoneum of female BALB/c mice were sorted and the same semiquantitative RT-PCR was conducted on the sorted samples as described in Figure 3.3. These data confirmed the expression data observed for L2 mice. Vcam1, Adamdec1, Spic and Hpgd were expressed in splenic but not in peritoneal B-1a cells. Message of Lpl, Mrc2 and Fpr1 was found in both peritoneal and splenic B-1a cells.



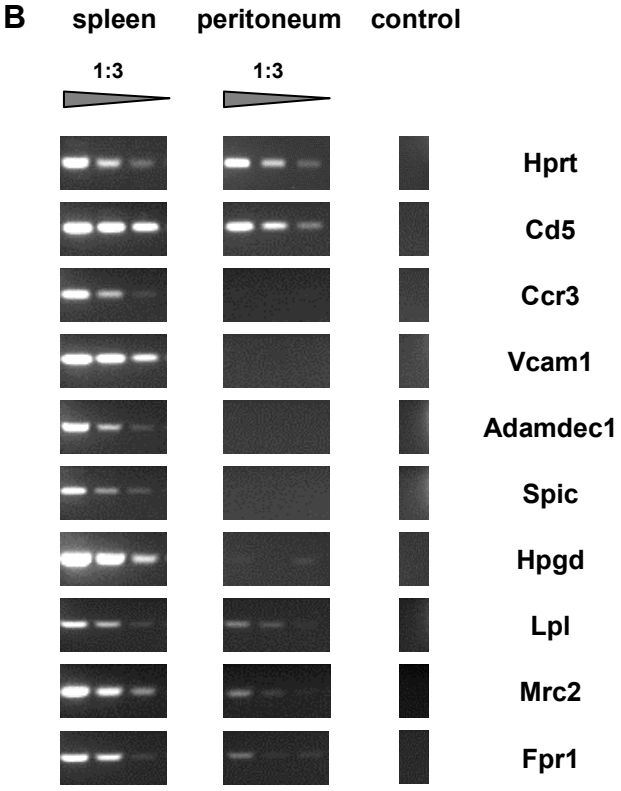


Figure 3.3. RT-PCR analysis of sorted B-1a cells from BALB/c mice. Spleen cells were sorted by IgM^+CD5^+ expression (purity: 98,4%), peritoneal B-1a cells were sorted by $IgM^{hi}IgD^{lo}CD5^+$ expression (purity: 98,6%). R4 represents gate for B-2 cells and reanalysis in R4 indicates that no B-2 cells were present in peritoneal B-1a samples.

3.1.1 Flow cytometrical analysis of VCAM-1 expression

Since for VCAM-1 antibodies are available, its expression was investigated at the protein level. Flow cytometrical analyses were performed on spleen and peritoneal cells of L2 mice and non-transgenic littermates. VCAM-1 expression was readily detected on gated splenic B-1a cells (Figure 3.4).

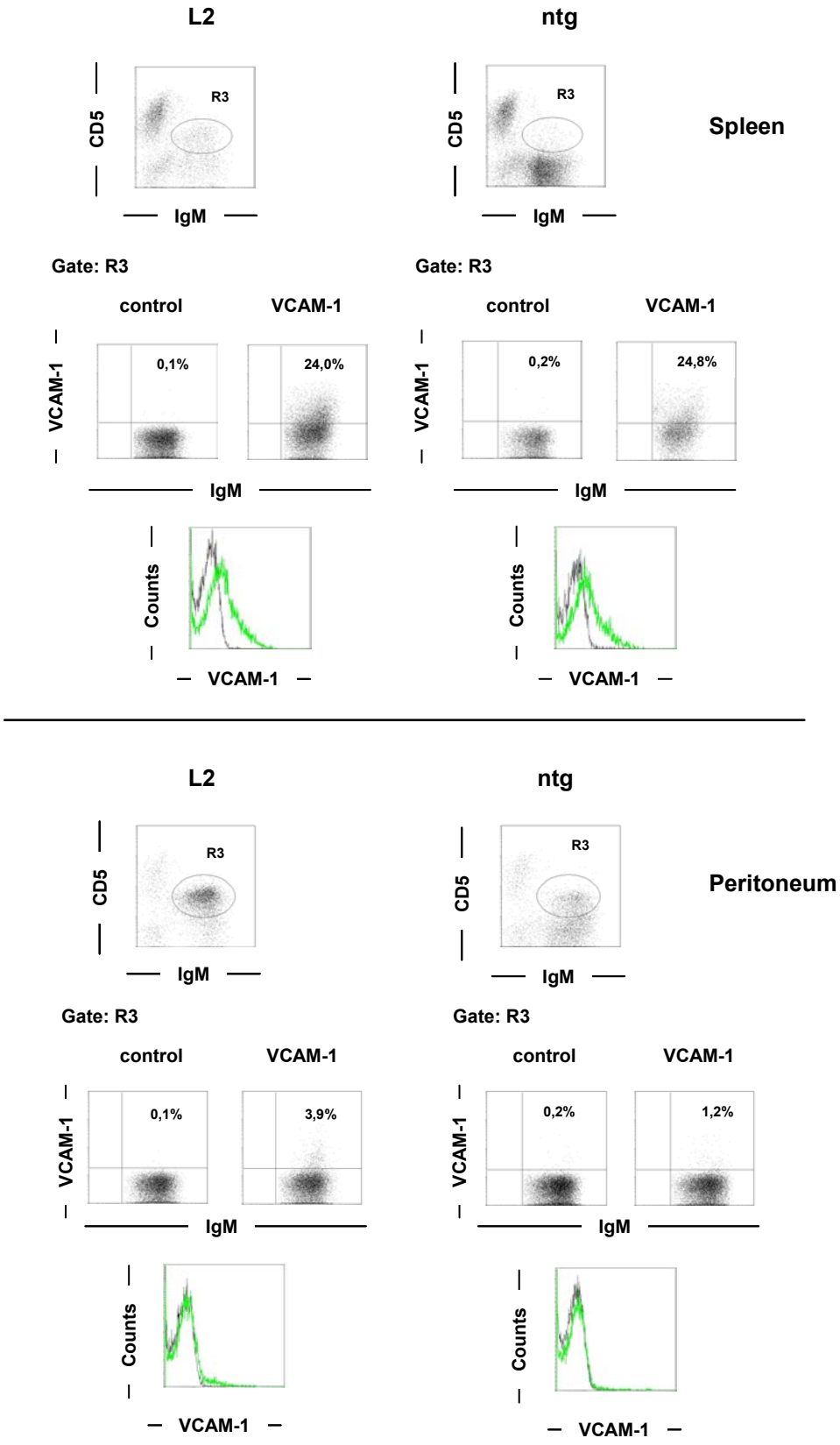


Figure 3.4. Flow cytometrical analysis of VCAM-1 expression on spleen and peritoneal cells from L2 mice and non-transgenic littermates. The staining was performed on pooled cells with 3 mice per group. Dot plots and histograms show cells gated by region 3 (R3). Black lines in histograms represent control staining and green lines staining with VCAM-1 antibody.

Interestingly, although no VCAM-1 expression was detected by RT-PCR, VCAM-1 positive cells could be found by flow cytometrical analysis of peritoneal IgM^+CD5^+ cells (Figure 3.4 and Table 3.1). The staining was apparently specific since it could be only marginally lowered by blockage of Fc-receptors.

Table 3.1. Analysis of VCAM-1 expression on peritoneal and splenic B-1a cells of L2 mice.

L2	VCAM-1 expression on gated IgM+CD5+ cells [% of gated][1]	
Mouse	peritoneum	spleen
1	6,0	46,6
2	5,9	48,6
3	4,4	31,2
4	4,7	41,8
Mean	5,3 ± 0,8	42,1 ± 7,8

[1] Table shows four independent stainings of individual L2 mice. All samples were treated with mouse serum and Fc-block before staining.

3.1.2 Intravenous transfer of peritoneal derived B-1 cells

First, the question was addressed of whether peritoneal B-1 cells could be influenced to express the respective marker genes after migration to the spleen. To this end cells obtained from peritoneal lavage of L2 mice were intravenously (i.v.) administered into $Rag1^{-/-}$ mice. This administration route ensures that the cells enter the spleen as well as the peritoneum. After 2,5 weeks cells from spleen and peritoneum of the transferred $Rag1^{-/-}$ mice (L2→$Rag1^{-/-}$) were sorted and analyzed by RT-PCR (Figure 3.5) and flow cytometry (Figure 3.6 and Figure 3.7).

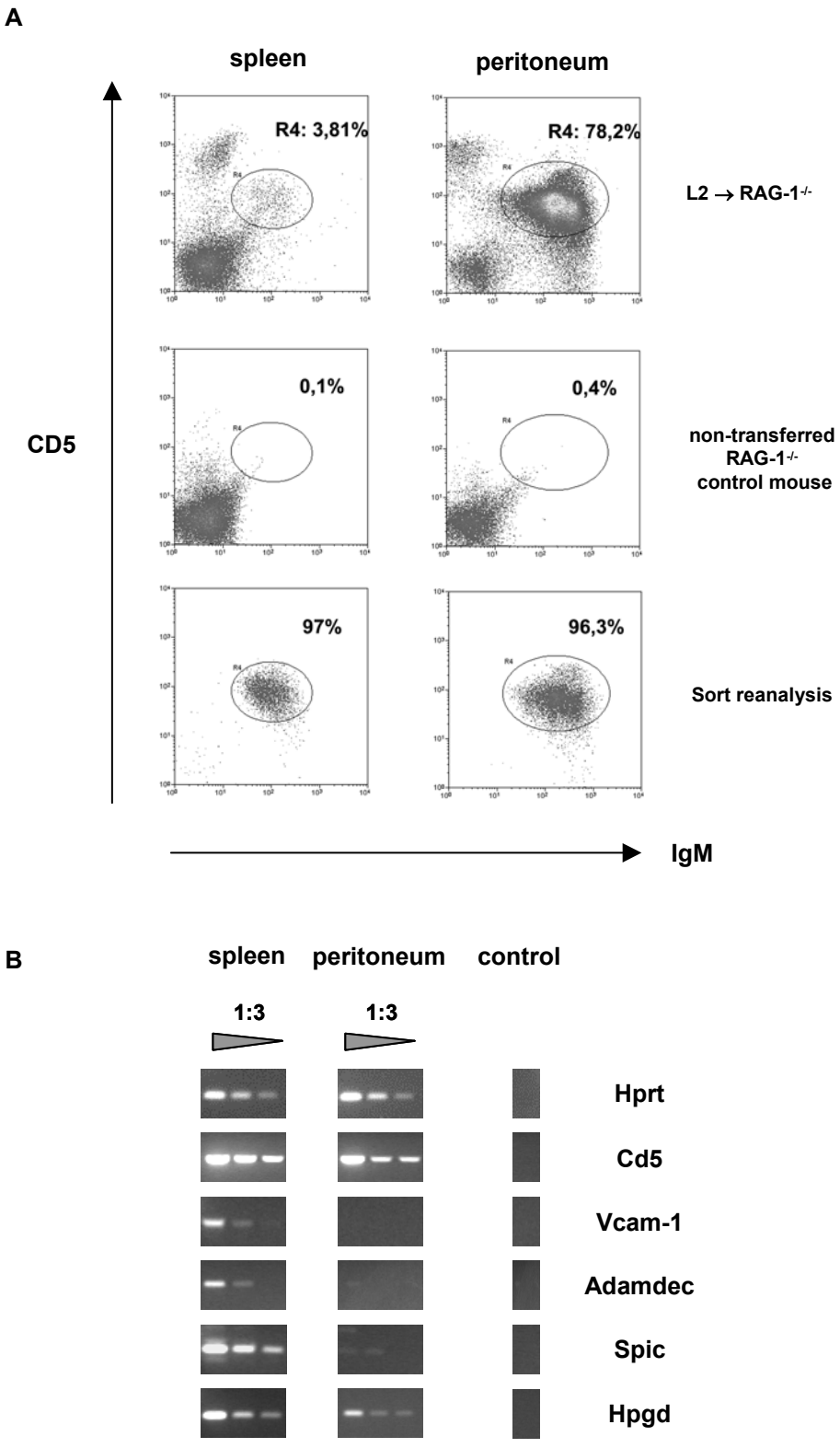
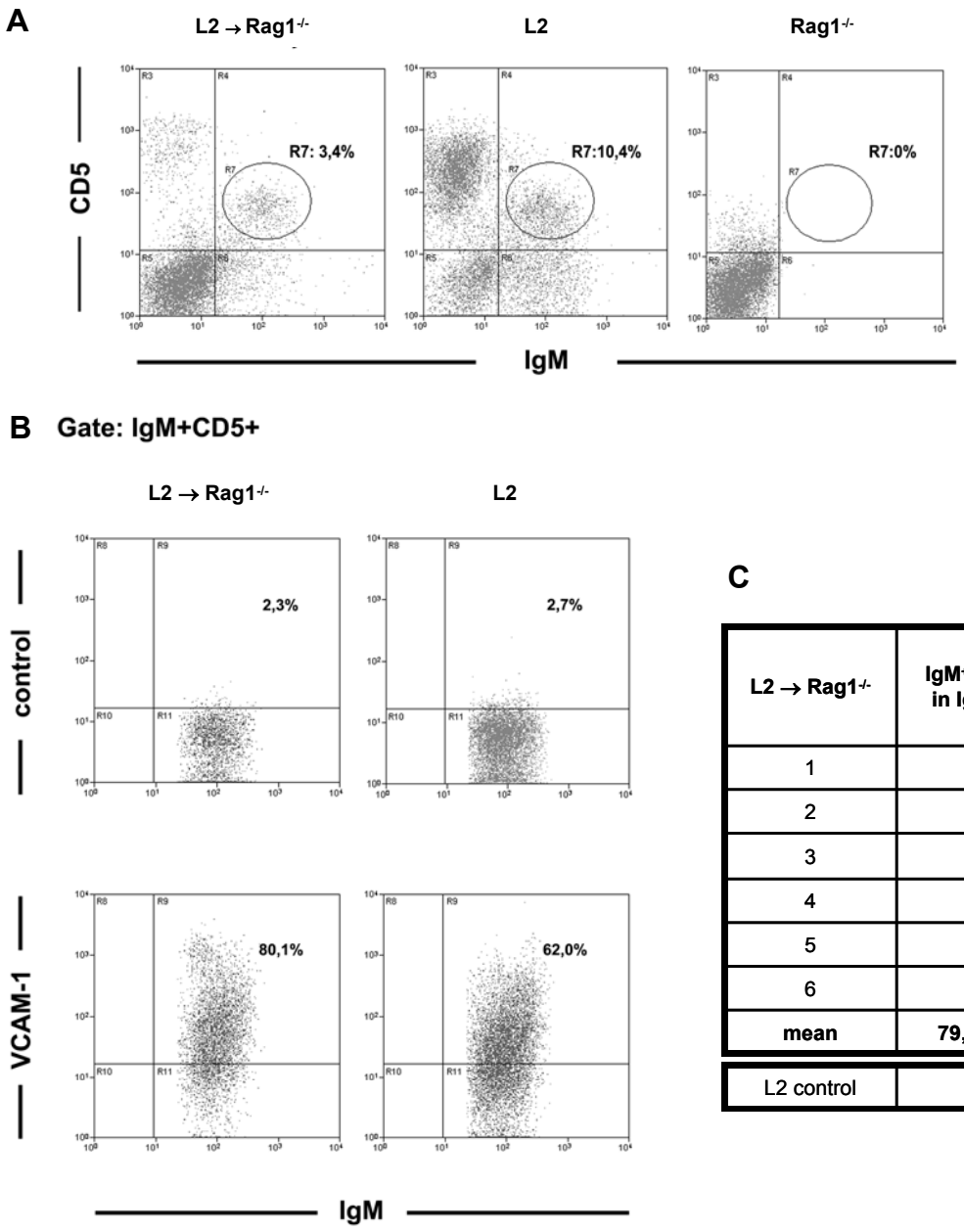


Figure 3.5. Cell sorting and semiquantitative RT-PCR analysis of cells from Rag1$^{-/-}$ mice transferred i.v. with cells from peritoneal lavages of L2 mice. A) Results of cells sorted for IgM^{+}CD5^{+} from peritoneum and spleen of transferred mice. Non-transferred Rag1$^{-/-}$ mice were included as staining controls. B) Semiquantitative RT-PCR analysis of sorted IgM^{+}CD5^{+} cells from A.

Analysis of L2 → Rag1$^{-/-}$ transfer i.v.: spleen

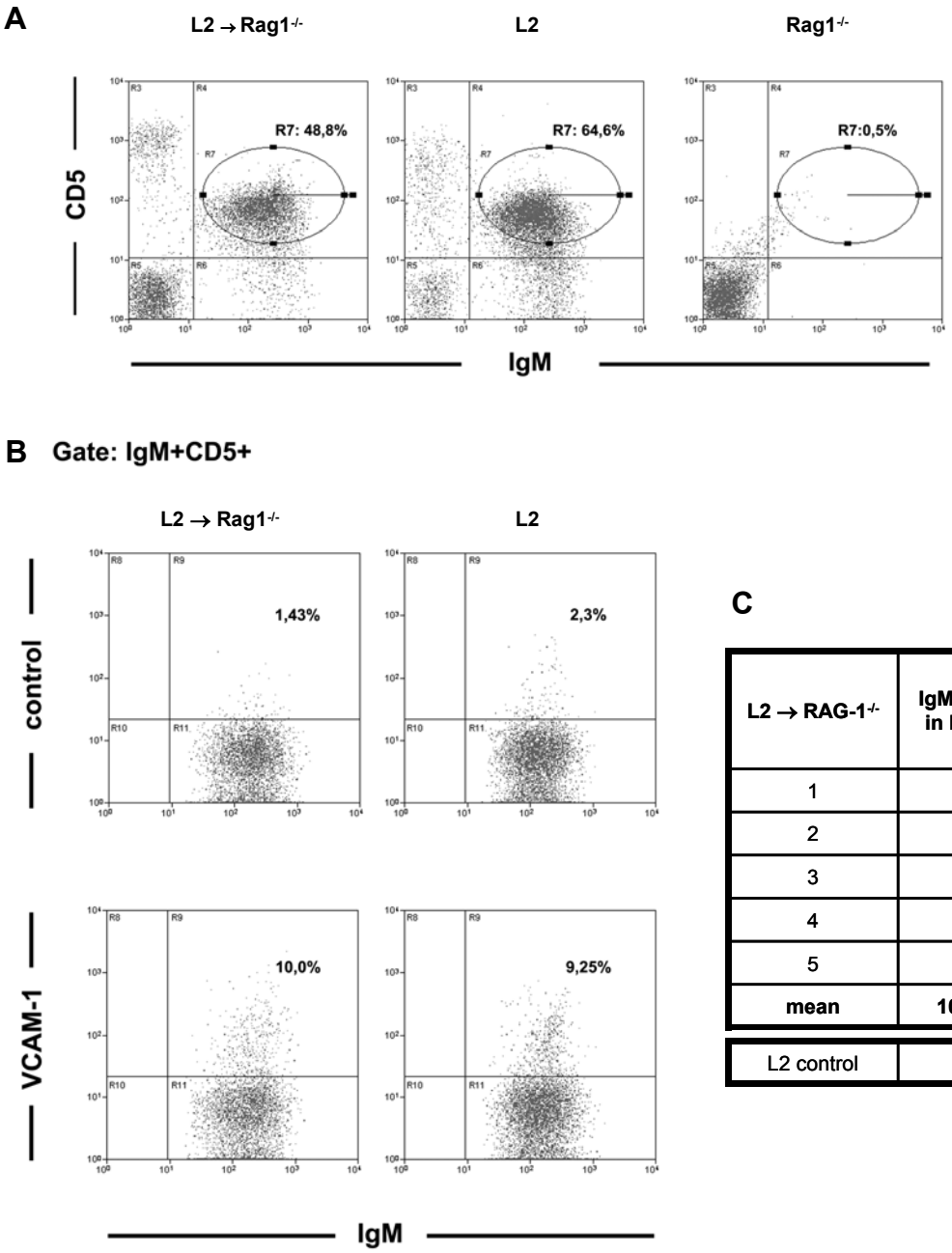


C

L2 → Rag1$^{-/-}$	% of IgM$^+$VCAM-1$^+$ in IgM$^+$CD5$^+$ gate
1	77,5
2	77,4
3	86,0
4	76,6
5	78,0
6	77,7
mean	**79,57 ± 4,0**
L2 control	59,53

Figure 3.6. Flow cytometrical analysis of VCAM-1 expression on spleen cells from Rag1$^{-/-}$ mice transferred i.v. with peritoneal cells from L2 mice. A) shows gated regions of splenic cells isolated from Rag1$^{-/-}$ recipient mice, an L2 control mouse and a non-transferred Rag1$^{-/-}$ control mouse. B) displays VCAM-1 stainings of cells from gate R7 and comparison with controls representing secondary reagent staining without addition of primary antibodies. Data shown in A and B represent the results from single mice. C) summarizes the results for VCAM-1 staining in IgM$^+$CD5$^+$ cells of six transferred individuals as well as an L2 control mouse after subtraction of background staining.

Analysis of L2 → Rag1$^{-/-}$ transfer i.v.: peritoneum



C

L2 → RAG-1$^{-/-}$	% of IgM$^+$VCAM-1$^+$ in IgM$^+$CD5$^+$ gate
1	12,1
2	10,8
3	11,1
4	10,6
5	8,6
mean	**10,6 ± 1,3**
L2 control	6,95

Figure 3.7. Flow cytometrical analysis of VCAM-1 expression on peritoneal cells from Rag1$^{-/-}$ mice transferred i.v. with peritoneal cells from L2 mice. A) shows gated regions of peritoneal cells isolated from Rag1$^{-/-}$ recipient mice, an L2 control mouse and a non-transferred Rag1$^{-/-}$ control mouse. B) displays VCAM-1 stainings of cells from gate R7 and comparison with controls representing stainings by secondary reagent without addition of primary antibodies. Data shown in A and B represent results from single mice. C) summarizes the results for VCAM-1 staining in IgM$^+$CD5$^+$ cells of five transferred individuals as well as an L2 control mouse after subtraction of background staining.

The spleen of transferred mice (Figure 3.6) contained IgM^+CD5^+ and low numbers of IgM^+CD5^- cells. $CD5^+IgM^-$ cells representing T cells were also seen. In RT-PCR (Figure 3.5) the marker genes Vcam-1, Spic and Adamdec1 were expressed in the splenic $CD5^+IgM^+$ cells of the L2→$Rag1^{-/-}$ mice while expression in the peritoneal cavity was low or absent. However, Hpgd message could be seen in these cells.

RT-PCR data for Vcam1 were confirmed when expression of the VCAM-1 protein was analyzed in flow cytometry. Protein expression was readily detectable in the splenic IgM^+CD5^+ cells of the transferred $Rag1^{-/-}$ mice (Figure 3.6 B and C). In comparison to the L2 control mice the expression level in spleen seemed even slightly elevated. In peritoneal cavity (Figure 3.7) similar levels of VCAM-1 protein expression were observed between transferred mice and control mice. Thus, peritoneal B-1 cells change to a splenic B-1 cell phenotype when residing in the spleen of the i.v. transferred $Rag1^{-/-}$ mice.

3.1.3 Peritoneal transfer of B-1 cells - kinetics

Next the question was addressed of whether peritoneal B cells also switch on the expression of the respective genes if administered via the peritoneal cavity, the site of their original location, and then enter the spleen. This should resemble more closely the situation found in the body when peritoneal B-1 cells migrate via the blood stream.

First, the kinetic of reconstitution was studied in order to find the optimal time point for the analysis of IgM^+CD5^+ cells of the spleen. Cells from peritoneal lavages of L2 mice were administered i.p. into $Rag1^{-/-}$ mice and single mice were analyzed at intervals for IgM^+CD5^+ cells in the spleen and peritoneum.

As it can be seen in Figure 3.8, the percentage of IgM^+CD5^+ cells in the spleen increased until 3 weeks after transfer and then remained constant. Similarly, the percentage of IgM^+CD5^+ cells in the peritoneal cavity peaked 3 weeks after transfer. After that time point cell numbers remained constant. Since the highest number of IgM^+CD5^+ cells was found in spleen and peritoneum 3 weeks after transfer, this time period was chosen for subsequent transfers.

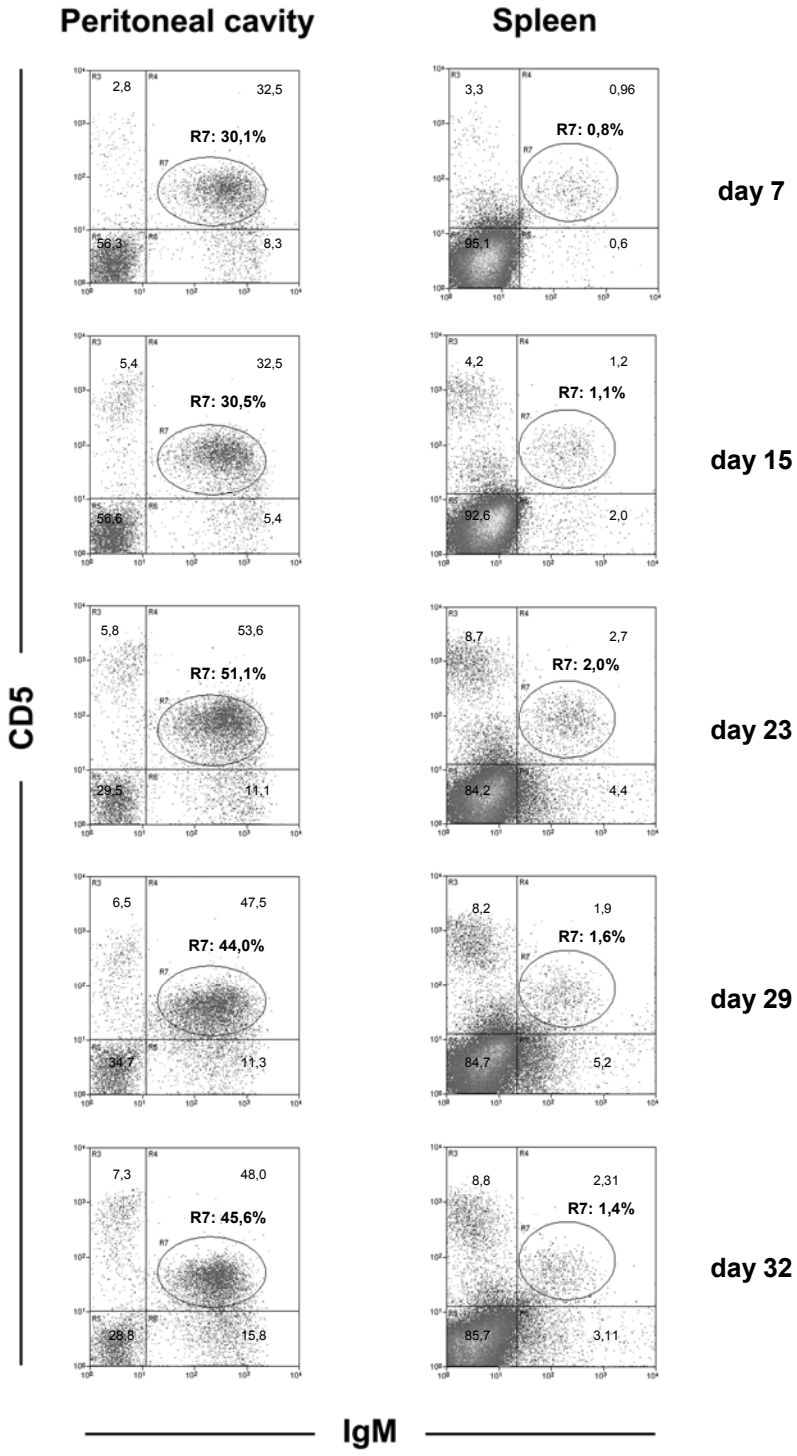


Figure 3.8. Kinetics of reconstitution of Rag1$^{-/-}$ mice intraperitoneally transferred with cells from peritoneal lavages of L2 mice. Dot plots are gated on lymphoid cells. Region 7 (R7) represents B-1a cells, percentage of IgM^{+}CD5^{+} cells in R7 is given. Additional numbers represent percentage of lymphoid cells in the respective quadrants of the dot plot.

During the establishment of the kinetics VCAM-1 expression was also followed up at the protein level in IgM^+CD5^+ cells. The splenic cells were positive for VCAM-1 surface staining at all time points investigated (Figure 3.9). This demonstrated the potential of these cells to upregulate VCAM-1 expression in B-1 cells that entered the spleen independent of the way of B-1 cell administration.

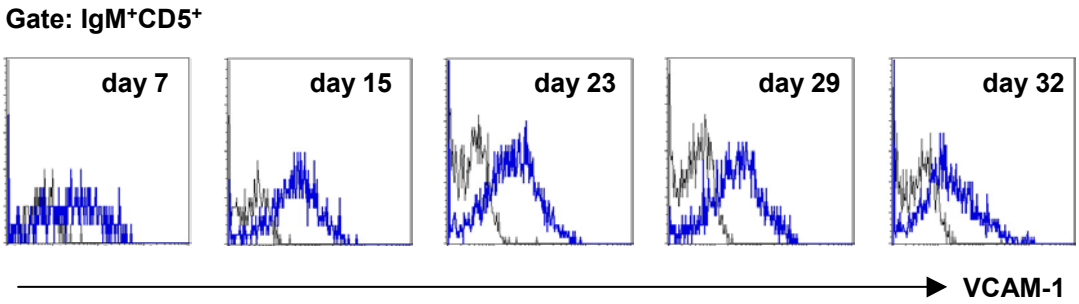


Figure 3.9. VCAM-1 expression on splenic IgM^+CD5^+ cells from $Rag1^{-/-}$ mice transferred with peritoneal cells of L2 mice. Time points of analysis are given in each histogram. Black lines represent controls, blue lines represent staining with VCAM-1 antibody.

To follow up whether also the other marker genes are expressed in the transferred B-1 cells, transfers were repeated. Peritoneal lavages from L2 mice were transferred i.p. into $Rag1^{-/-}$ mice and 3 weeks after transfer cells from spleen and peritoneum of these mice were isolated and pooled. Samples were then split. One fraction from both organs was sorted for IgM^+CD5^+ expression and used in RT-PCR. The other fraction was used for flow cytometrical analyses concerning VCAM-1 expression.

As already indicated by the VCAM-1 staining performed during the transfer kinetic Vcam1 message was detected in the IgM^+CD5^+ spleen cells from the transferred mice. Also Adamdec1, Spic and Hpgd were expressed (Figure 3.10). In contrast only slight expression of Hpgd and Spic could be observed in the peritoneal cavity while message of Vcam1 and Adamdec1 was absent.

Similarly, flow cytometrical analysis of spleen cells from mice transferred with peritoneal cells revealed VCAM-1 protein expression on gated splenic IgM^+CD5^+ cells (Figure 3.11).

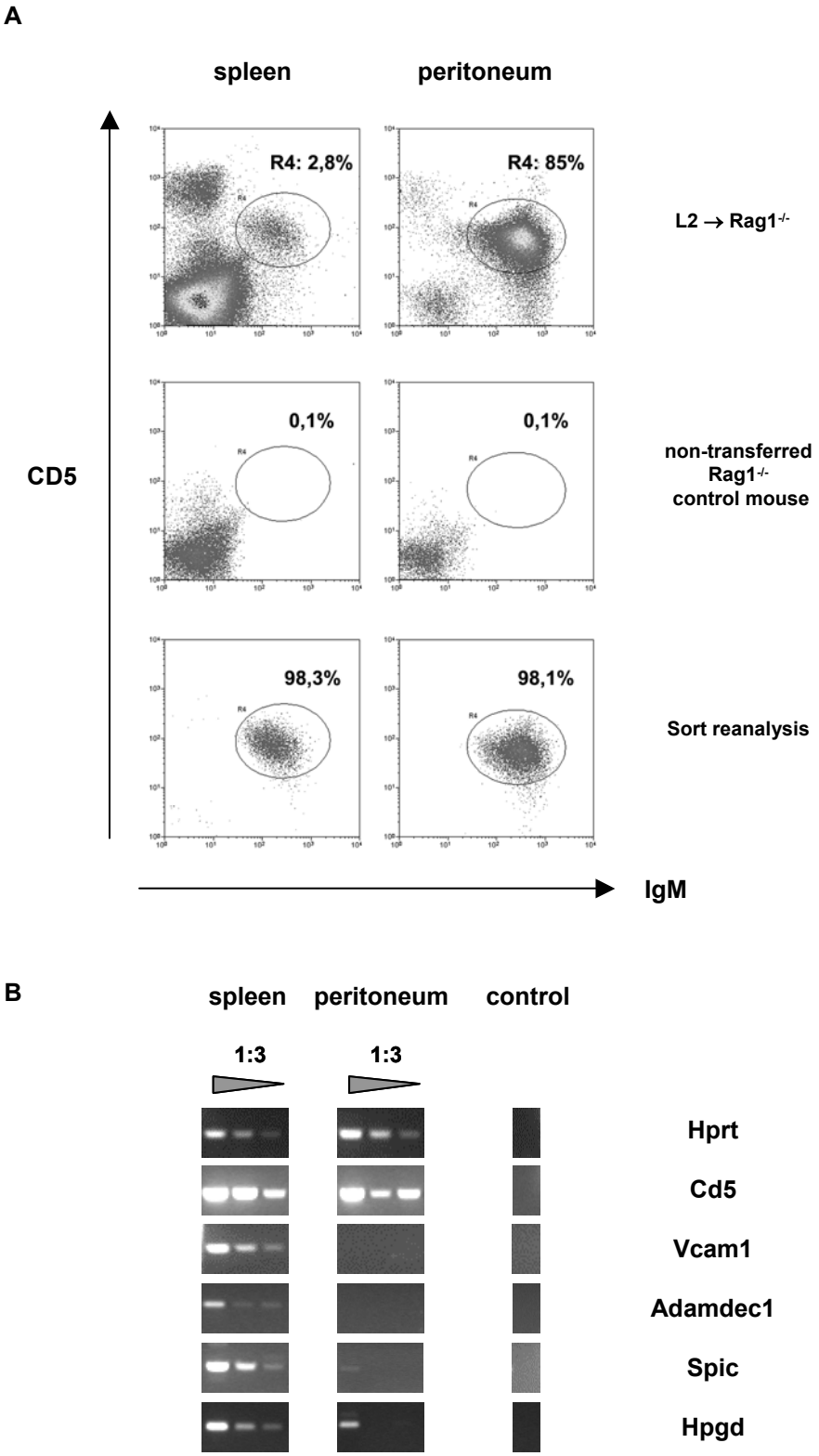
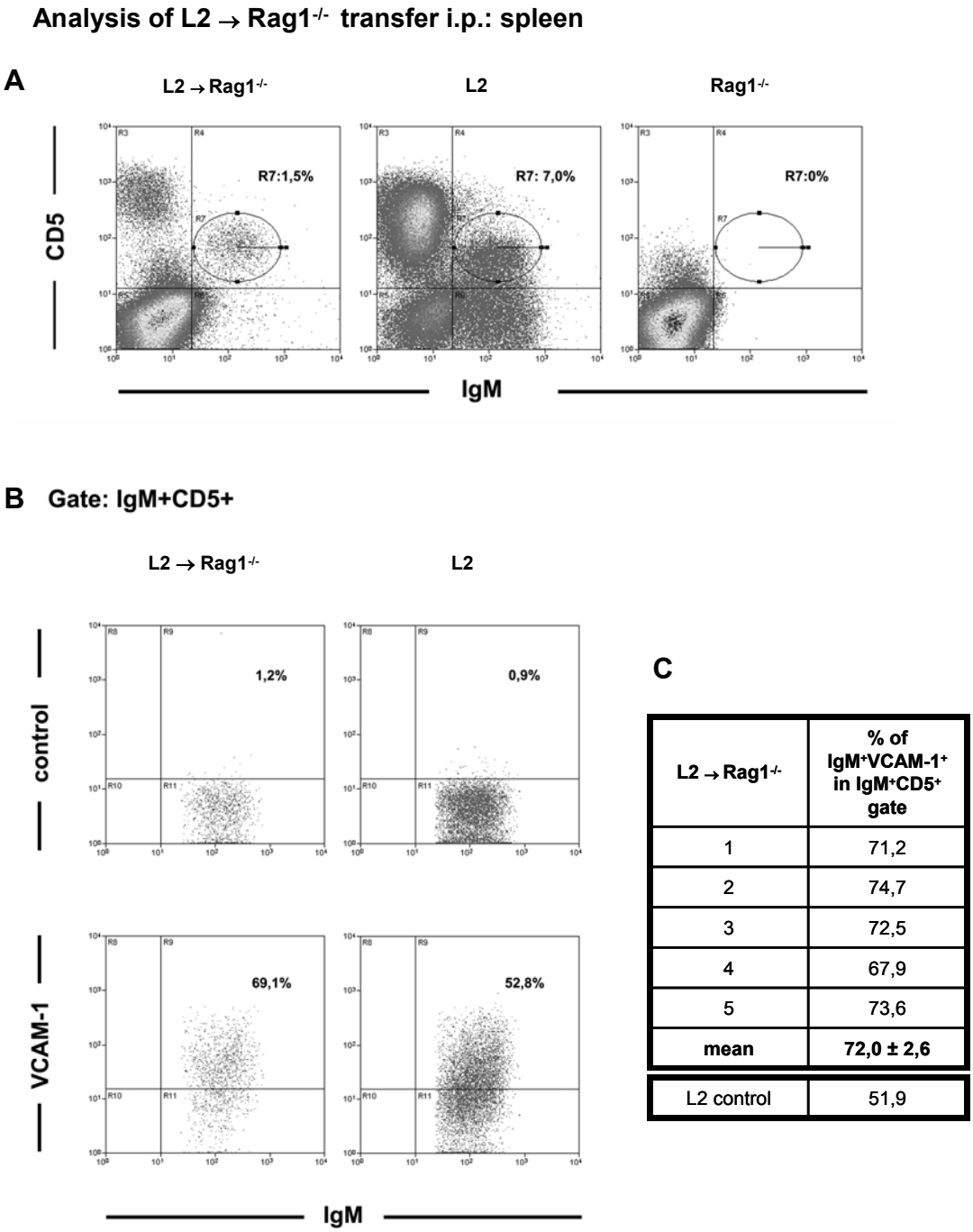


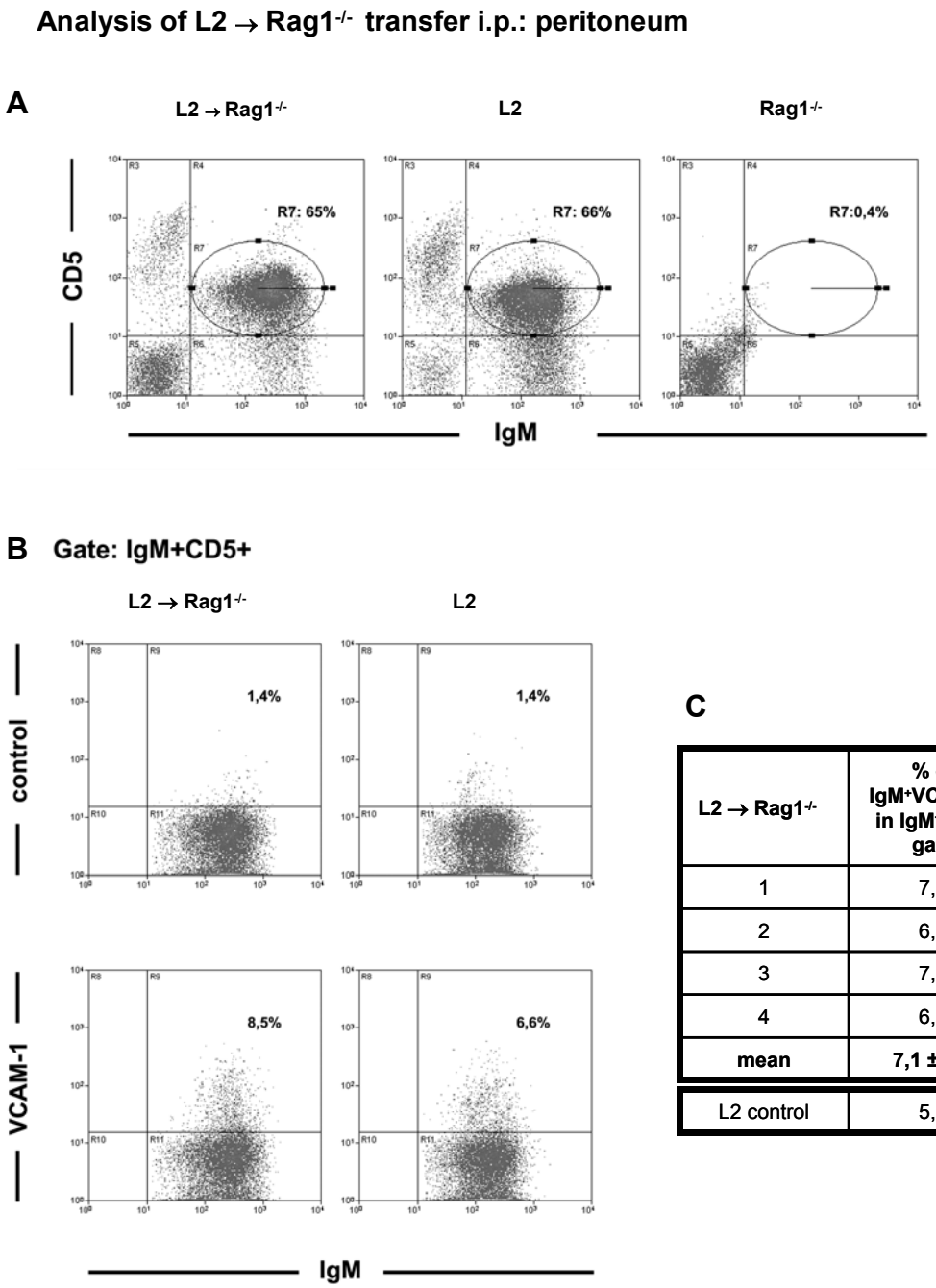
Figure 3.10. Cell sorting and analysis of IgM$^+$CD5$^+$ cells from peritoneum and spleen of Rag1$^{-/-}$ deficient mice transferred i.p. with peritoneal cells of L2 mice. A) Sort and reanalysis of sorted cells. B) Semiquantitative RT-PCR with samples from A.



C

L2 → Rag1$^{-/-}$	% of IgM$^+$VCAM-1$^+$ in IgM$^+$CD5$^+$ gate
1	71,2
2	74,7
3	72,5
4	67,9
5	73,6
mean	**72,0 ± 2,6**
L2 control	51,9

Figure 3.11. Flow cytometrical analysis of spleen cells of Rag1$^{-/-}$ mice intraperitoneally transferred with peritoneal lavages from L2 mice. A) shows gated regions of splenic cells isolated from a Rag1$^{-/-}$ recipient, an L2 control mouse and a non-transferred Rag1$^{-/-}$ control mouse. B) displays VCAM-1 stainings of cells from gate R7 and comparison with controls representing stainings with secondary reagent without addition of primary antibodies. Data shown in A) and B) represent results from single mice. C) summarizes the results for VCAM-1 staining in IgM$^+$CD5$^+$ cells of five transferred individuals as well as an L2 control mouse after subtraction of background staining.



L2 → Rag1$^{-/-}$	% of IgM$^+$VCAM-1$^+$ in IgM$^+$CD5$^+$ gate
1	7,6
2	6,9
3	7,1
4	6,8
mean	**7,1 ± 0,3**
L2 control	5,2

Figure 3.12. Flow cytometrical analysis of peritoneal cells of individual Rag1$^{-/-}$ transfer mice transferred i.p. with peritoneal lavages from L2 mice. A) shows gated regions of peritoneal cells isolated from a Rag1$^{-/-}$ recipient, an L2 control mouse and a non-transferred Rag1$^{-/-}$ control mouse. B) displays VCAM-1 stainings of cells from gate R7 and comparison with controls representing secondary reagent without addition of primary antibodies. Data shown in A) and B) represent results from single mice. C) summarizes the results for VCAM-1 staining in IgM$^+$CD5$^+$ cells of four transferred individuals as well as an L2 control mouse after subtraction of background staining.

Flow cytometrical analysis of cells isolated from the peritoneal cavity of the Rag1$^{-/-}$ mice transferred with peritoneal cells from L2 mice displayed a low frequency of cells expressing VCAM-1 amongst the IgM^{+}CD5^{+} gated cells while in RT-PCR analysis no expression could be observed (e.g. Figure 3.10 and Figure 3.12). This discrepancy of protein and mRNA expression of VCAM-1 was observed reproducibly in all transfer experiments. Similarly, as shown above, in L2 mice no RT-PCR product could be obtained from peritoneal B-1a cells while VCAM-1 expressing cells were detected in flow cytometry (Figure 3.4). This discrepancy was now further investigated. Since only low numbers of VCAM-1 protein positive cells were present in the IgM^{+}CD5^{+} B cell population it was possible, that the RT-PCR performed was not sensitive enough to detect VCAM-1 message within this cell population.

Therefore, CD19^{+} peritoneal B-1 cells from L2 mice were sorted for VCAM-1 protein expression (Figure 3.13). The reanalysis showed that the sorted sample contained more than 90% of cells positive for VCAM-1 surface expression. These cells were then subjected to RT-PCR to test for the presence of Vcam1 message. Interestingly, no Vcam1 specific cDNA could be observed even at high PCR cycle numbers (Figure 3.13). This was true for two independent primer pairs used. Thus, in peritoneal B-1 cells VCAM-1 protein expression without expression of mRNA is observed. One explanation could be that these cells represent a population of migratory cells which have down-regulated Vcam1 expression after entry of the peritoneal cavity but still display the protein on the surface.

3.1.4 Transfer of VCAM-1 negative peritoneal B-1 cells

The sorted CD19^{+} cells that were negative for surface VCAM-1 protein were used in an additional transfer experiment (for reanalysis see Figure 3.13). To exclude an effect due to the absence of T cells in the sorted population the transfers also included peritoneal lavage cells of Ig$\alpha^{-/-}$ mice. These mice are devoid of B cells due to a block of B cell development (Pelanda et al., 2002). Thus, all peritoneal cell populations including T cells are found in these mice except B cells. Due to the low numbers of sorted cells only two Rag1$^{-/-}$ mice could be transferred. Therefore, sorting cells from transferred mice to obtain samples for RT-PCR was not possible since two mice would not supply sufficient amounts of cells. Hence, the mice were analyzed by flow cytometry.

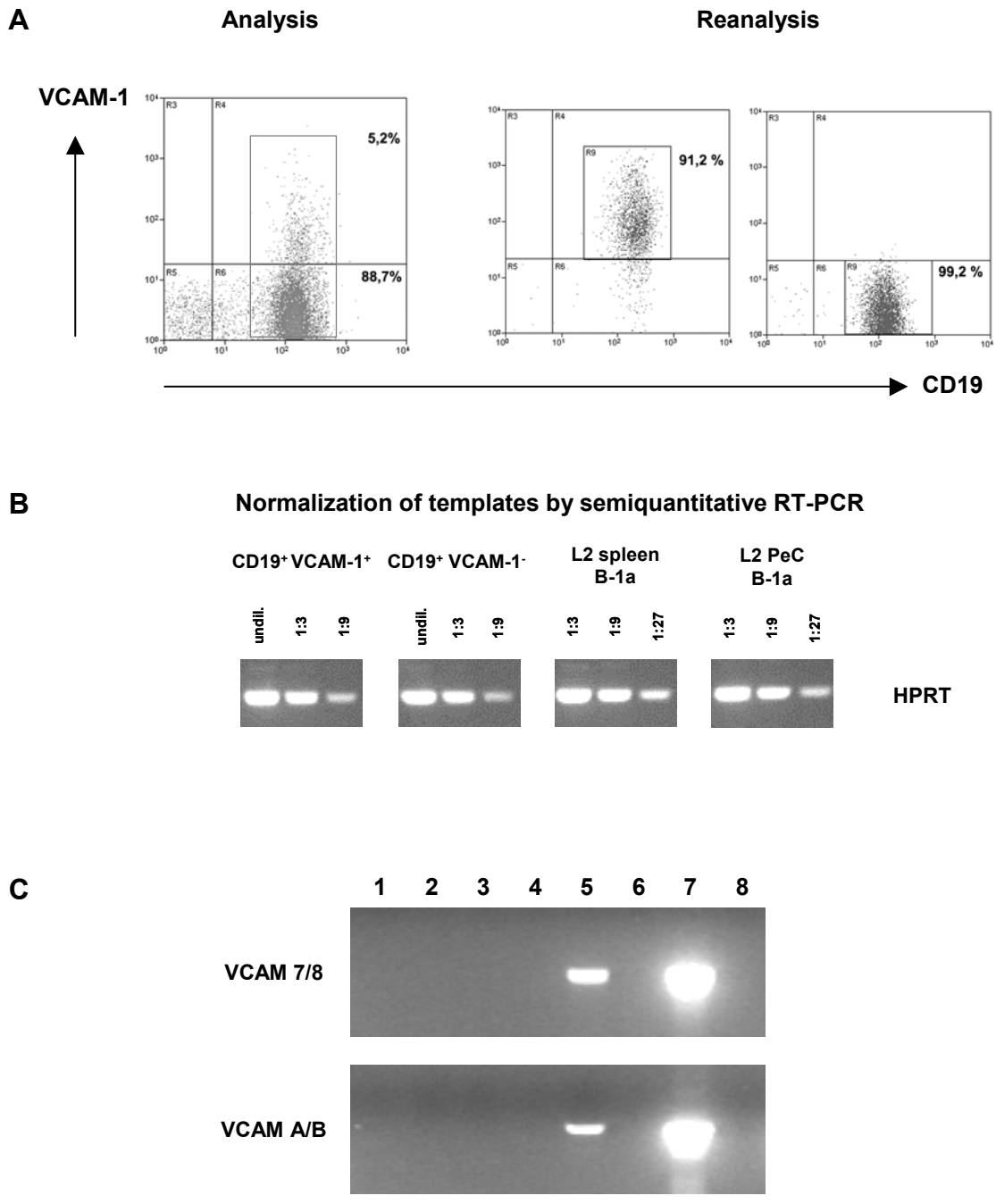


Figure 3.13. Analysis of Vcam1 expression in peritoneal CD19$^+$ cells of L2 mice. A) The left dot plot shows peritoneal cells stained for VCAM-1 and CD19 before sorting. The two dot plots on the right show reanalyses of VCAM-1$^+$CD19$^+$ cells or VCAM-1$^-$CD19$^+$ cells, respectively. B) cDNA from sorted cell samples shown in A was prepared and normalized by semiquantitative RT-PCR according to expression of the housekeeping gene HPRT. C) Samples with the respective normalized highest amounts of cDNA were tested for Vcam1 expression by PCR using different intron-spanning primer pairs (VCAM 7/8 and VCAM A/B) and 38 cycles during PCR. cDNA-Templates: lane 1: CD19$^+$VCAM-1$^+$ undiluted sample; lane 2: CD19$^+$VCAM-1$^+$ undiluted control (without reverse transcriptase (RT)); lane 3: CD19$^+$VCAM-1$^-$ undiluted sample; lane 4: CD19$^+$VCAM-1$^-$ undiluted control (without RT); lane 5: B-1a cells from spleen of L2 mice (1:3 dilution); lane 6: B-1a cells from peritoneal cavity of L2 mice (1:3 dilution); lane 7 positive control (total spleen cells); lane 8: water control.

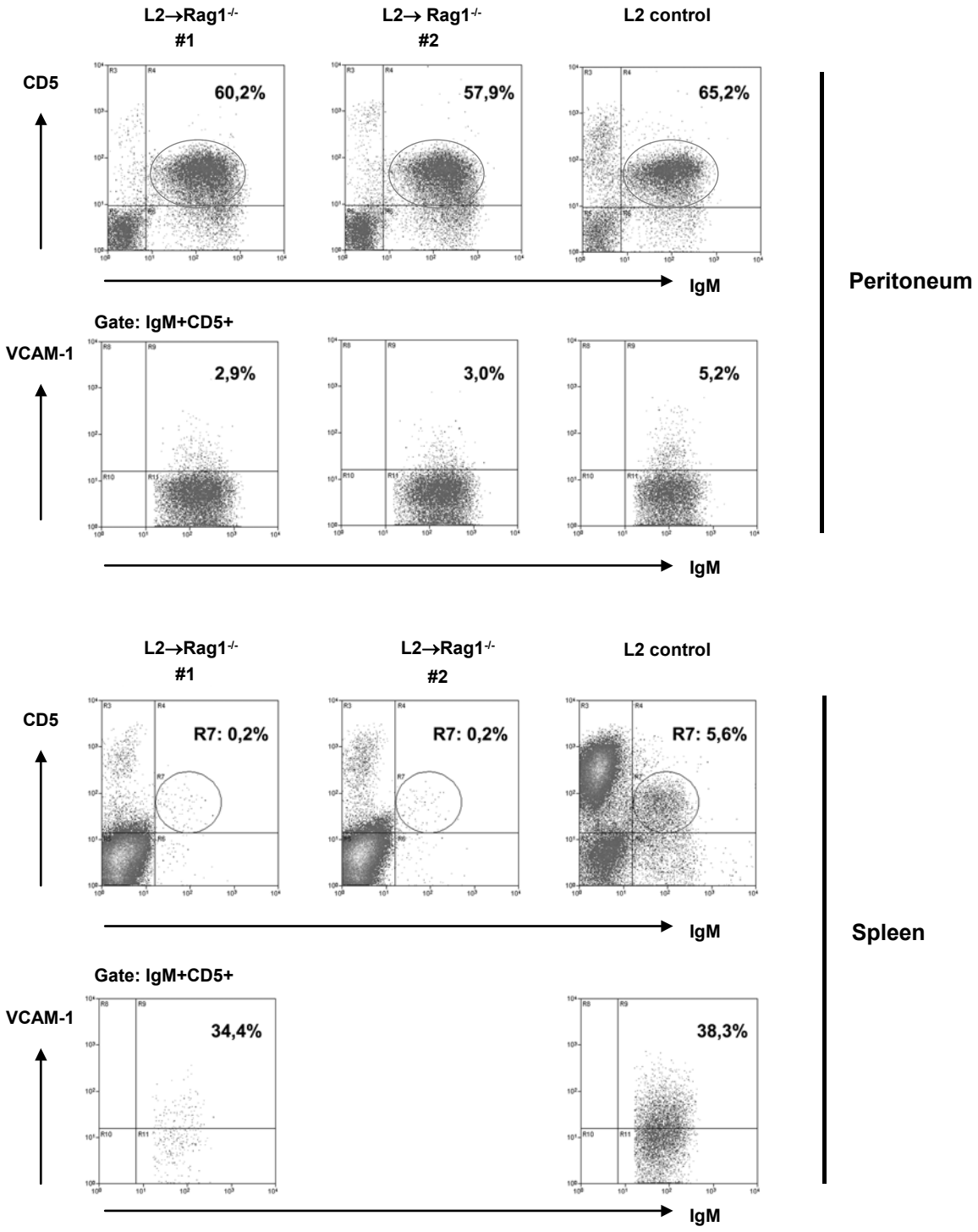


Figure 3.14. Flow cytometrical analysis of cells from peritoneum and spleen of Rag1$^{-/-}$ mice transferred with peritoneal CD19^{+}VCAM-1^{-} cells from L2 mice. Transfers included a cotransfer of cells from peritoneal lavages of Ig$\alpha^{-/-}$ mice to provide T cell help if necessary. Two individual mice were analyzed two weeks after transfer. Peritoneal and splenic cells from an L2 mouse were stained as control. Rag1$^{-/-}$ control mice showed no IgM positive cells (data not shown). Spleen sample with VCAM-1 staining of the second L2 →Rag1$^{-/-}$ mouse was not determined for technical reasons.

VCAM-1 expression comparable to that found in L2 control mice could be detected in splenic IgM^+CD5^+ cells of the recipient mice (Figure 3.14). This result confirmed the intrinsic potential of peritoneal IgM^+CD5^+ cells to upregulate the expression of VCAM-1. VCAM-1 expression was also found on the IgM^+CD5^+ cells in the peritoneal cavity of the recipient mice. This might further support the idea that these cells belong to a population that has gained VCAM-1 protein expression while migrating into different sites of the body and after reentry of the peritoneal cavity downregulate Vcam1 gene expression.

3.1.5 Analysis of VCAM-1 deficient mice

VCAM-1 is an immunoglobulin-like transmembrane adhesion molecule that effects lymphocyte function in many ways. Thus it plays a role in lymphocyte migration (Cook-Mills, 2002), in rescue of germinal center B cells or thymocytes from apoptosis (Lindhout et al., 1993; Koopman et al., 1994; Koopman et al., 1997; Zaitseva et al., 1998) and might function in maturation and costimulation of T cells (Burkly et al., 1991; Damle et al., 1992; Schlegel et al., 1995). However, VCAM-1 in these examples is expressed by endothelial, epithelial or follicular dendritic cells. The impact on lymphocytes is mediated via interaction with integrin VLA-4 ($\alpha4\beta1$), the major ligand of VCAM-1, which is expressed by the lymphocytes.

In contrast only few examples for VCAM-1 expression by lymphocytes themselves exist. Thus thymocytes and apoptotic T cells were shown to express VCAM-1 (Ishiyama et al., 1998). In B cells, VCAM-1 protein expression was demonstrated on blood derived human peripheral B lymphocytes and in a human plasmacytoma at the RNA and protein level (Xia et al., 2001). However, nothing is known so far about the function of VCAM-1 expressed by the B cells. In the context with the finding that B-1 cells in spleen express VCAM-1 the impact of its expression on B cells was further investigated in this work.

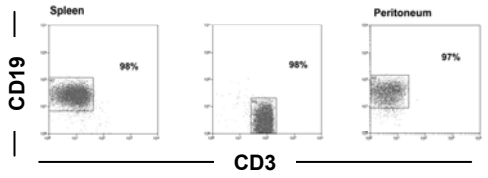
For this purpose a mouse model that was supposed to delete VCAM-1 specifically in B cells was studied. Mice with loxP-flanked (floxed) conditional Vcam1 null alleles were crossed with mice expressing the Cre protein under the control of the B cell specific CD19 promoter. Homozygous mice for the floxed Vcam1 alleles and heterozygous for CD19cre were termed flox/flox; cre/+. Non-transgenic littermates with respect to Cre-expression were termed flox/flox; +/+.

First, the deletion of Vcam1 alleles in B cells of flox/flox; cre/+ mice was tested. To this end $CD19^+$ B cells were sorted from spleen and peritoneum of these mice and analyzed by Southern blot. Additionally, $CD3^+$ T cells were isolated from the same samples by cell sorting and included as negative controls (Figure 3.15). Most of the $CD19^+$ B cells showed a deletion of the functional alleles whereas $CD3^+$ T cells kept the floxed alleles in an undeleted state. However, faint bands for the non-deleted alleles were detected in the $CD19^+$ fraction of the Southern blot.

Next, flow cytometrical analyses of B cell compartments from flox/flox; +/+ control mice and flox/flox; cre/+ mice was carried out on pooled cell populations (Figure 3.16). A twofold reduction of $IgM^{hi}IgD^{lo}$ B-1 cell numbers (Figure 3.16 A, R2) and a fourfold reduction of B-1a cell numbers could be observed (Figure 3.16 A, R3) in the peritoneal cavity of flox/flox; cre/+ compared to flox/flox; +/+ mice. The proportion of the IgD^+IgM^{lo} B-2 cells in the peritoneum, however, was nearly the same (Figure 3.16 A, R1). In the spleen, the follicular B cell compartment was similar in flox/flox; +/+ and flox/flox; cre/+ mice and similar proportions of marginal zone B cells were detected (Figure 3.16 B; R4 and R5). Furthermore, no differences between IgM^+CD5^+ cells in the spleens of both strains could be observed (Figure 3.16 B, R3).

To assess the significance of these data the stainings were repeated with individual mice (see Figure 3.17 and Figure 3.18). Control mice bearing wildtype alleles for Vcam1 and the CD19 Cre transgene (+/+; cre/+) were included since Sato and colleagues had demonstrated that the development of B-1 cells in CD19 deficient mice is severely decreased (Sato et al., 1996b). Thus, the observed reduction of B-1 cells could be due to the CD19 deficiency in one allele of the flox/flox; cre/+ mice. However, since only a few of these mice were obtained from the breedings, analysis of only three +/+;cre/+ mice could be performed within the given time frame (see Figure 3.17 and Figure 3.18). The reduction of peritoneal B-1 and B-1a cells could be observed in all flox/flox; cre/+ mice (Figure 3.17 tables B, C). However, this reduction was also observed in the +/+;cre/+ control mice. Thus, the reduction is most likely due to the lower level of CD19 expression and not due to the lack of VCAM-1 expression.

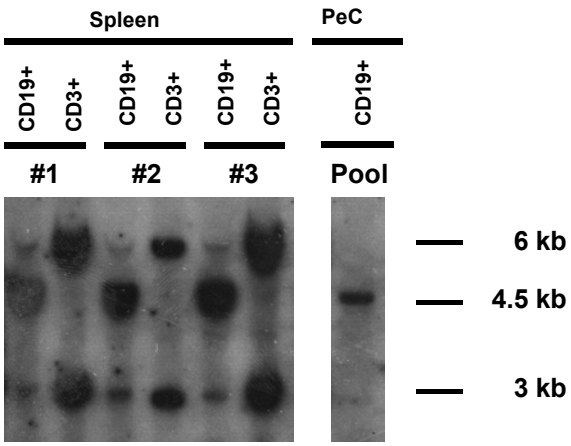
A



B

Mouse	Population	Purity [%]
	spleen	
#1	CD19+	97,4
	CD3+	97,9
#2	CD19+	98,3
	CD3+	97,9
#3	CD19+	97,5
	CD3+	98,4
	peritoneum	
Pool #1-#3	CD19+	97,0

C



D

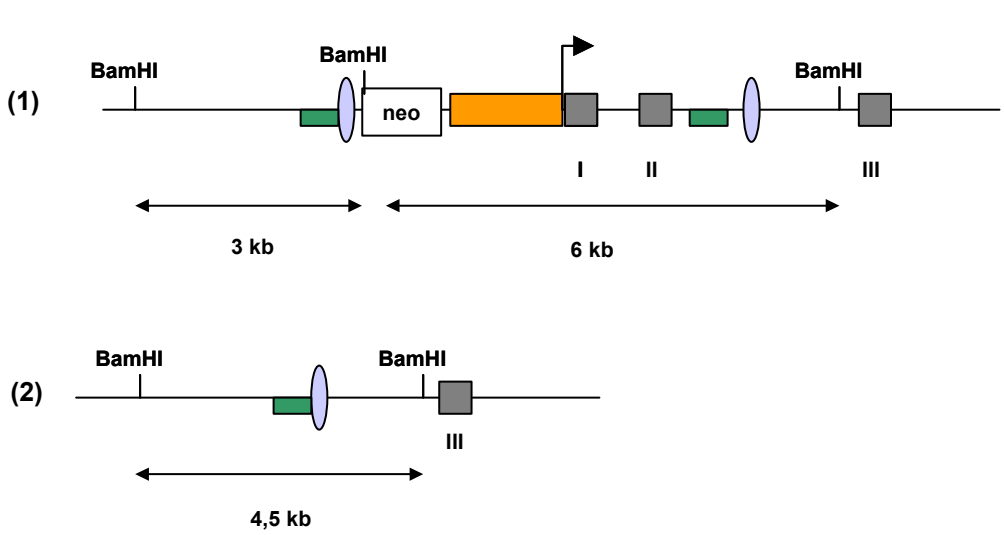


Figure 3.15. Isolation of CD3$^+$ T cells and CD19$^+$ B cells from flox/flox; cre/+ mice and Southern blot on sorted samples. Three mice were analyzed individually. A) Reanalyses of sorted splenic B and T cells from mouse #2 and from CD19$^+$ sorted pooled peritoneal cells from mice #1-3 (dot plot on the right). B) Summary for all sorted samples. C) Southern blot of sorted samples. DNA isolated from the respective samples was digested with BamHI and hybridized with a radiolabeled Vcam1 specific probe. It consists of a mixture of two *Bgl*II/ *Spe*I restriction fragments. Floxed alleles yield two restriction fragments at 6 kb and 3 kb, deleted alleles yield a 4,5 kb restriction fragment. D) Schematic map of the 5' part of the targeted Vcam1 locus (according to Terry et al., 1997) . LoxP sites are displayed as blue ovals, promoter is shown as orange box, exons are indicated by gray boxes and roman numbers. Green boxes indicate hybridization sites for the Vcam1 specific probe.

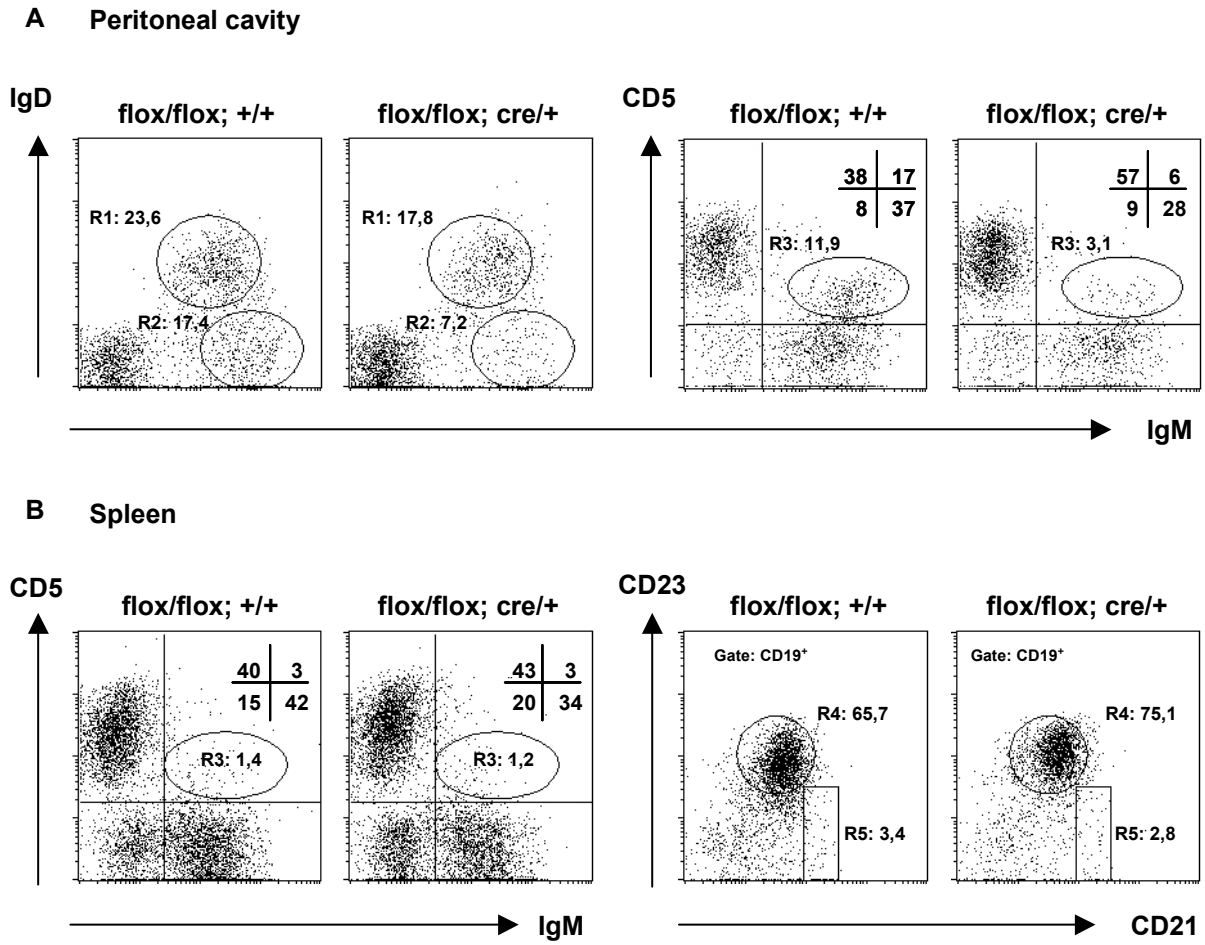


Figure 3.16. Flow cytometrical analysis of B cell subsets in flox/flox; +/+ and flox/flox; cre/+ mice. A) Peritoneal staining. R1: $IgD^{+}IgM^{+}$ B-2 cells; R2: $IgD^{lo/neg}IgM^{hi}$ B-1 cells; R3: B-1a cells. B) Splenic staining. R3: B-1a; R4: follicular B-2; R5: marginal zone B cells. Stainings represent pooled populations from 3 mice.

B-2 cell numbers in the peritoneal cavity were comparable between flox/flox; +/+ and flox/flox; cre/+ mice (Figure 3.17). Similarly, in the spleen of these mice no significant differences could be detected in the B cell compartments between the different mouse types (Figure 3.18). To extend these studies, also VCAM-1 expression on B cells was tested in these mice. Surprisingly, no difference in VCAM-1 expression on cells from flox/flox; +/+ and flox/flox; cre/+ mice gated for IgM^{+} could be observed (Figure 3.19) in independent experiments.

B-2

A	[%] of **IgD^{+}IgM^{+}**		
	flox/flox; +/+	flox/flox; cre/+	+/+; cre/+
1	15,4	16,6	24,5
2	21,7	16,7	38,1
3	32,1	33,1	29,7
4	16,0	17,2	n.d.
5	19,3	14,3	n.d.
6	15,0	19,1	n.d.
Mean	**19,9 ± 6,5**	**19,5 ± 6,8**	**30,8 ± 6,9**

B-1

B	[%] of **IgD^{lo}IgM^{hi}**		
	flox/flox; +/+	flox/flox; cre/+	+/+; cre/+
1	38,5	18,5	13,2
2	21,8	11,0	11,8
3	22,2	12,2	23,9
4	22,4	13,0	n.d.
5	25,8	11,7	n.d.
6	37,5	10,7	n.d.
Mean	**28,0 ± 7,9**	**12,9 ± 2,9**	**16,3 ± 6,6**

Peritoneal cavity

B-1a

C	[%] of **IgM^{+}CD5^{+}**		
	flox/flox; +/+	flox/flox; cre/+	+/+; cre/+
1	26,9	7,7	8,1
2	10,3	5,6	5,8
3	14,7	8,6	14,7
4	21,6	8,3	n.d.
5	21,8	8,2	n.d.
6	29,3	6,3	n.d.
Mean	**20,8 ± 7,2**	**7,5 ± 1,2**	**9,5 ± 4,6**

Figure 3.17. Flow cytometrical analysis of peritoneal cells isolated from individual flox/flox; cre/+ mice and littermate control mice. Mean values and standard deviations of the mean from each set are given at the bottom of each table. Results are derived from two independent experiments. Mice 1-3 experiment 1; mice 4-6 experiment 2.

To confirm these results, splenic cells from individual flox/flox; +/+ and flox/flox; cre/+ mice were sorted and analyzed by RT-PCR for Vcam1 mRNA (Figure 3.20). For this purpose, a forward primer binding in exon 1 and a reverse primer binding in exon 3 of the Vcam1 gene were used. Thus, the forward primer lies within the deletable region of the floxed Vcam1 alleles (for overview see schematic map Figure 3.15).

Spleen

B-1a

D	[%] of IgM$^+$CD5$^+$		
	++/--	++/+-	--/++
1	1,72	3,6	1,58
2	1,89	2,6	1,66
3	2,43	2,3	2,5
4	1,05	1,24	n.d.
5	1,89	0,55	n.d.
6	1,68	1,21	n.d.
Mean	**1,8 ± 0,4**	**1,9 ± 1,1**	**1,9 ± 0,5**

E	[%] of IgM$^+$CD5$^-$		
	++/--	++/+-	--/++
1	57,3	42,3	53,3
2	51,7	49,9	45,0
3	47,4	56,1	43,9
4	60,7	60,9	n.d.
5	57,3	58,4	n.d.
6	51,3	60,5	n.d.
Mean	**54,3 ± 4,9**	**54,7 ± 7,3**	**47,4 ± 5,1**

FO

F	[%] of CD19$^+$CD23$^+$CD21$^+$		
	++/--	++/+-	--/++
1	74,3	82,2	77,5
2	76,5	82,1	81,7
3	76,9	82,2	85,8
4	68,8	75,1	n.d.
5	73,3	74,2	n.d.
6	70,6	76,3	n.d.
Mean	**73,4 ± 3,2**	**78,7 ± 3,9**	**81,7 ± 4,2**

MZ

G	[%] of CD19$^+$CD23^{lo}CD21$^+$		
	++/--	++/+-	--/++
1	3,3	4,1	2,2
2	3,7	3,8	2,2
3	5,2	4,2	1,1
4	7,6	3,0	n.d.
5	3,0	5,3	n.d.
6	4,9	2,8	n.d.
Mean	**4,6 ± 1,7**	**3,9 ± 0,9**	**1,8± 0,6**

Figure 3.18. Flow cytometrical analysis of spleen cells isolated from individual flox/flox; cre/+ mice and littermate control mice. Mean values and standard deviations of the mean from each set are given below the results for individual mice. Results were derived from two independent experiments. Mice 1-3 experiment 1; mice 4-6 experiment 2.

Vcam1 expression was detectable in both samples. Thus, both mouse types contain cells in which the floxed part of the Vcam1 gene was not deleted by CD19 controlled Cre. This might explain the lack of a phenotype in the flox/flox; cre/+ mice.

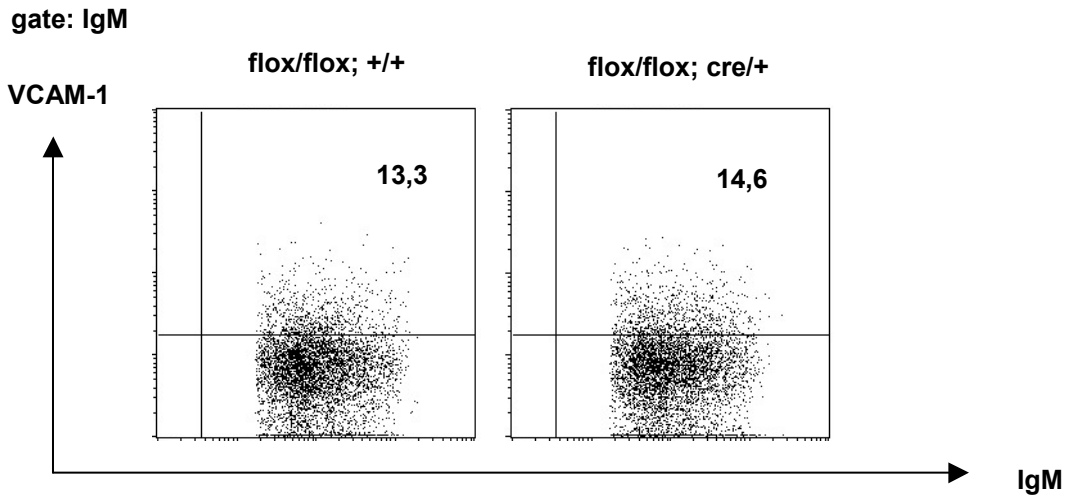


Figure 3.19. Dot plots showing VCAM-1 expression on IgM$^+$ gated spleen cells of flox/flox; +/+ and flox/flox; cre/+ mice. Dot plots are representative for a VCAM-1 staining of two individual mice (total mice analyzed: n=3 per group). Numbers indicate percentage of VCAM-1$^+$ B cells in comparison to non-stained controls. On average VCAM-1 was expressed in 14,2% ± 0,8 of splenic IgM$^+$ cells in the tested flox/flox; +/+ mice and 15,1% ± 2,5 in flox/flox; cre/+ mice.

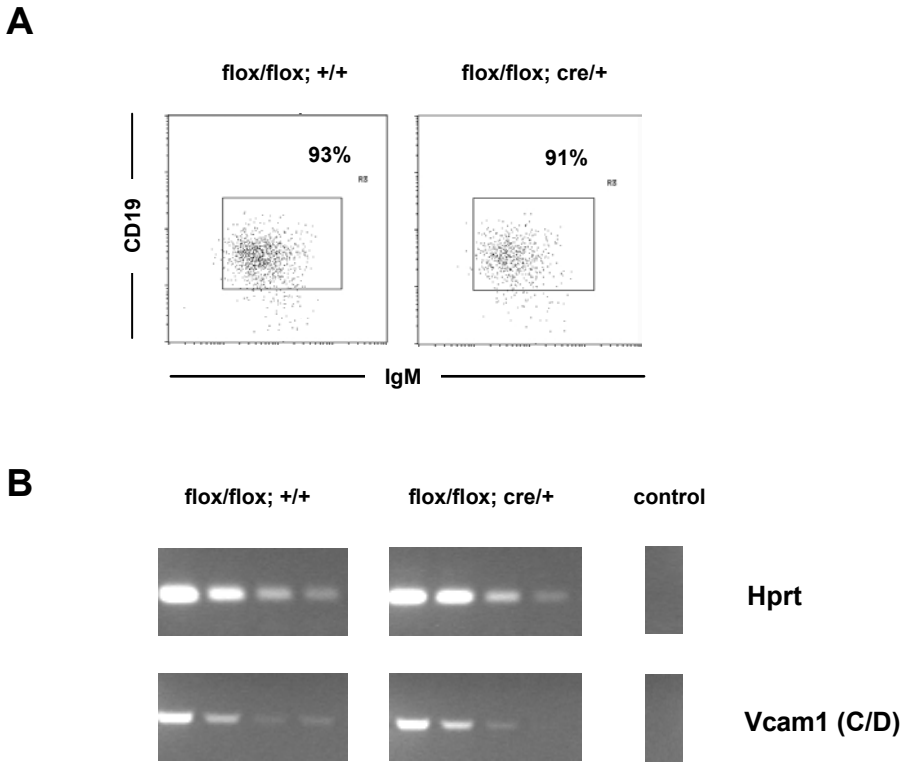


Figure 3.20. Sort and analysis of spleen cells from flox/flox; +/+ and flox/flox; cre/+ mice. A) Reanalysis of CD19$^+$IgM$^+$ sorted samples from spleen of indicated mice from VCAM-1$^{flox/flox}$/CD19cre breedings. B) Semiquantitative RT-PCR on sorted samples from A. The used forward primer (C) for Vcam1 PCR binds in exon 1, the reverse primer (D) in exon 3. Samples were normalized upon expression of the housekeeping gene Hprt.

3.2 Group 2 genes

The data obtained from the expression arrays were also used in order to find molecules specifically expressed in B-1 cells. Candidate genes that were expressed in B-1 cell populations in spleen as well as peritoneum but absent in B-2 cells were determined and classified into group 2 by these criteria. The expression array data were screened strictly for this purpose: First, only genes expressed in B-1 cells of both L2 and BALB/c mice were accepted and second, the mean average difference values for gene expression in B-2 cells had to be at background level, i.e. at 61, indicating the absence of respective gene expression in these cells.
Four candidate genes were obtained by this stringent selection (Table 3.2) and first investigated by RT-PCR in order to confirm the array data.

Table 3.2. Group 2 genes representing candidate genes for B-1 cell specific markers.

		L2						**BALB/c**					
		Peritoneum				***Spleen***		***Peritoneum***		***Spleen***			
Gene (Protein)	**Affy id**	**B-1a**	*SD*[2]	**B-1b**	*SD*	**B-1a**	*SD*	**B-1a**	*SD*	**B-1a**	*SD*	**B-2**	*SD*
F11r (Junction cell adhesion molecule 1, JAM1)	103816_at	**808**[1]	38	**1089**	107	**584**	153	**1283**	291	**439**	216	**61**	1
Adml (ADM, Adrenomedullin)	102798_at	**683**	49	**491**	80	**1327**	470	**986**	229	**1267**	332	**61**	0
Api6 (Apoptosis inhibitory 6)	93445_at	**133**	31	**274**	175	**733**	89	**393**	109	**1024**	829	**61**	1
Wee1 (Wee-1 like protein kinase)	101458_at	**505**	55	**266**	60	**361**	79	**184**	87	**503**	155	**61**	1

[1] Number indicates the mean average difference value.
[2] SD = standard deviation of mean average difference value of three replicate experiments.

By this analysis only two genes still fulfilled the above criteria (F11r and Adml) while Wee1 and Api6 no longer could be classified into group 2 (Figure 3.21 and Figure 3.22). Wee1, the Affymetrix gene designation for a wee-1 like kinase, showed weak expression in peritoneal and splenic B-1a cells of L2 mice (Figure 3.21). However,

when B cells from normal BALB/c mice were tested expression was found in peritoneal and splenic B-1a cells but at an equal level also in B-2 cells (Figure 3.21). Api6, expressing a protein termed CD5 antigen-like precursor, SP-alpha or CT-2, was strongly expressed in splenic B-1a cells and weakly in peritoneal B-1a cells from L2 mice. In BALB/c mice Api6 was expressed at similar levels in peritoneal and splenic B-1a cells as well as in B-2 cells (Figure 3.21).

Adml and F11r fulfilled the criteria for group 2 in RT-PCR analysis. Both genes were strongly expressed in peritoneal and splenic B-1a cells from L2 and normal mice (Figure 3.22). A weak signal could be detected in the normalized cDNA from splenic B-2 cells. However, this was attributed to the extreme sensitivity of the RT-PCR. These data were tried to verify by flow cytometry. Unfortunately, in preliminary experiments using the commercially available antibody against Adrenomedullin no signal could be obtained in intracellular stainings of B-1 cells.

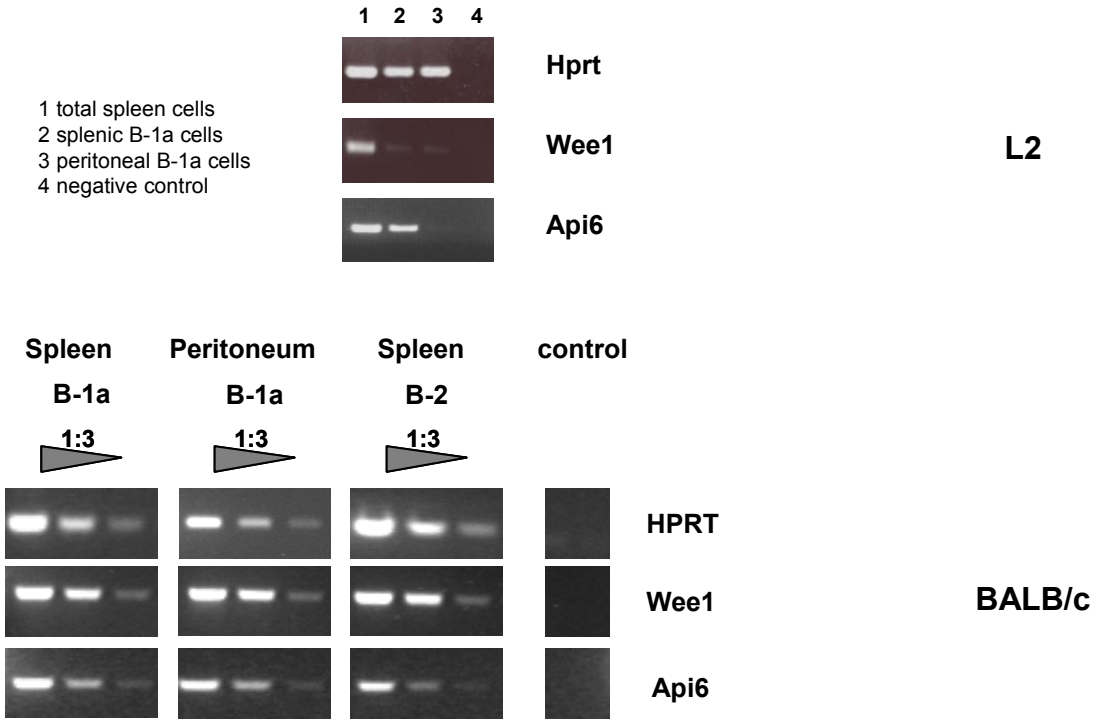


Figure 3.21. RT-PCR for Wee1 and Api6 on B-1a cells from L2 and BALB/c mice. B-1a cells from peritoneum and spleen have been sorted $IgM^{+}CD5^{+}$ and B-2 cells from spleen have been sorted $IgM^{lo}CD5^{neg}$.

On the other hand, JAM-1, encoded by the F11r gene, could be clearly detected on peritoneal B-1a cells from L2 as well as BALB/c mice (Figure 3.23, R1). In addition, peritoneal B-1b cells could be stained for JAM-1 in L2 mice (Figure 3.23). All other peritoneal populations were negative for JAM-1 expression.

The splenic IgM^+CD5^+ B-1a population of L2 mice was also positive for JAM-1 staining (Figure 3.24) as well as IgM^+CD5^+ B-1a cells in the spleen of BALB/c mice. However, the JAM-1 staining in the splenic B-1a cells of BALB/c mice was apparently weak. Although a slight shift between control cells and cells stained for JAM-1 could be seen it is not as pronounced as in splenic B-1a cells of L2 mice.

Taken together these results show, that JAM-1 protein expression in the peritoneal cavity is detected on B-1 cells of L2 and normal mice. Thus, at this site it can be used as a B-1 cell specific marker for such cells. In the spleen of L2 mice JAM-1 can also be used as a marker for the IgM^+CD5^+ B-1a cells.

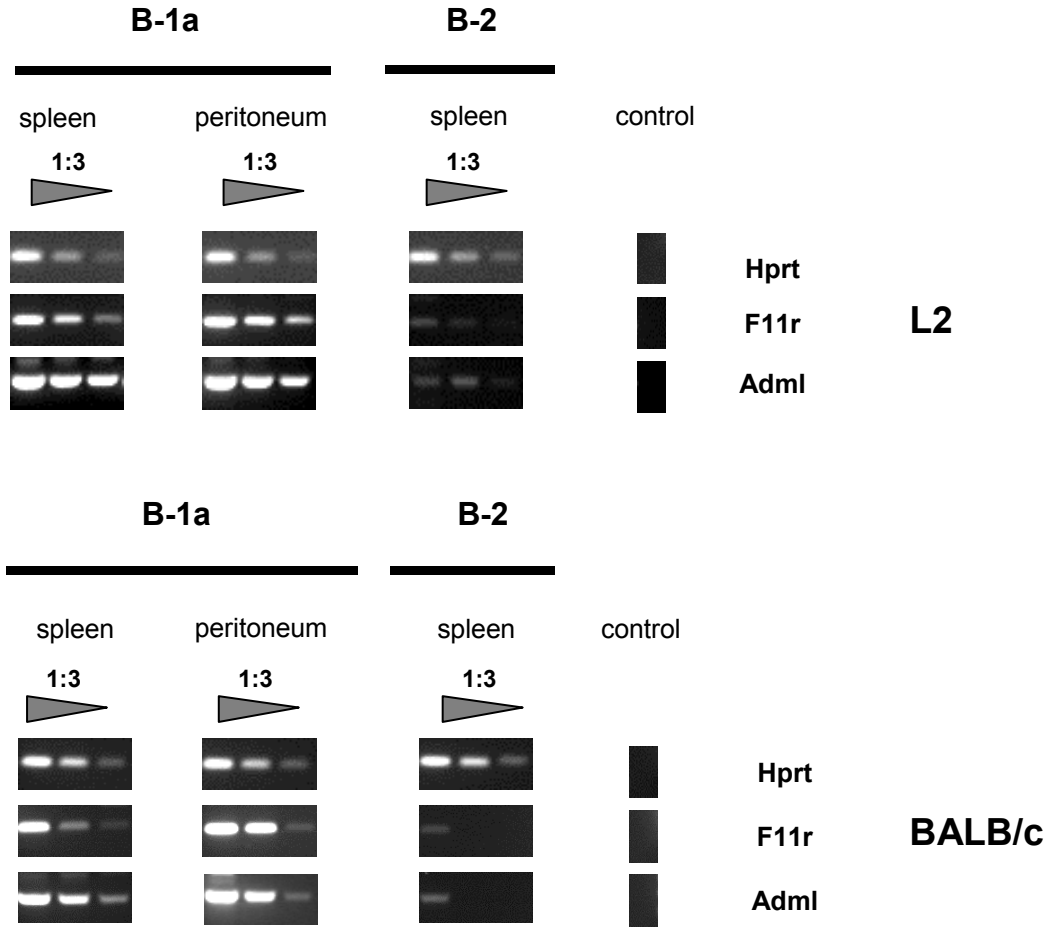


Figure 3.22. Semiquantitative RT-PCR for F11r and Adml on sorted cell populations from L2 and BALB/c mice. B-1a cells from peritoneum and spleen have been sorted as IgM^+CD5^+ and B-2 cells from spleen as $IgM^{lo}CD5^{neg}$.

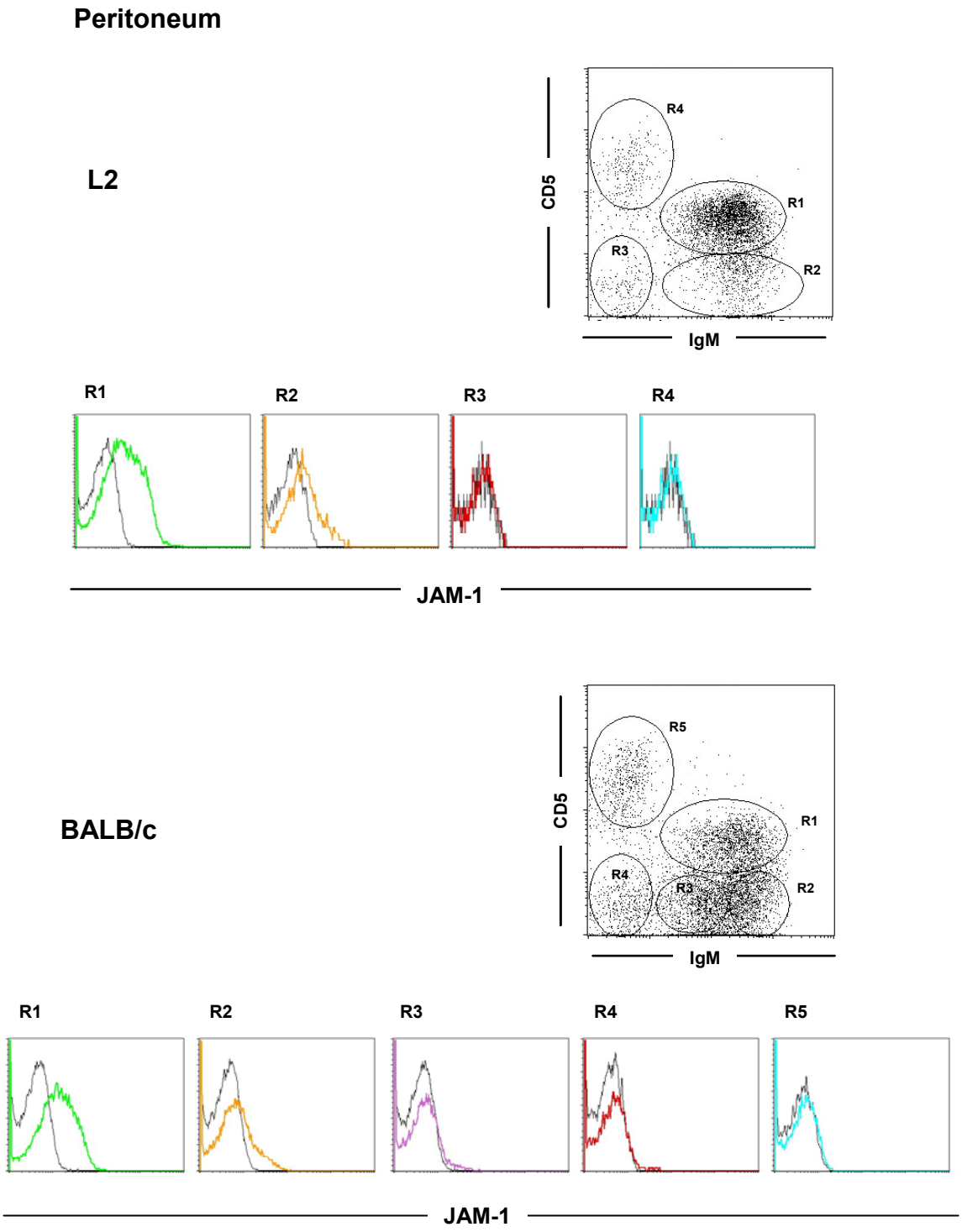


Figure 3.23. Flow cytometrical analysis of peritoneal cells for expression of JAM-1. Histogram overlays show JAM-1 expression in the following regions: L2 mice: R1: B-1a cells; R2: B-1b cells; R3: double negative cells; R4: T cells. BALB/c: R1: B-1a cells; R2: $IgM^{hi}CD5^{neg}$ containing B-1b cells; R3: $IgM^{lo}CD5^{-}$ containing B-2 cells; R4 double negative cells, R5: T cells. Black lines indicate control staining, colored lines represent JAM-1 staining.

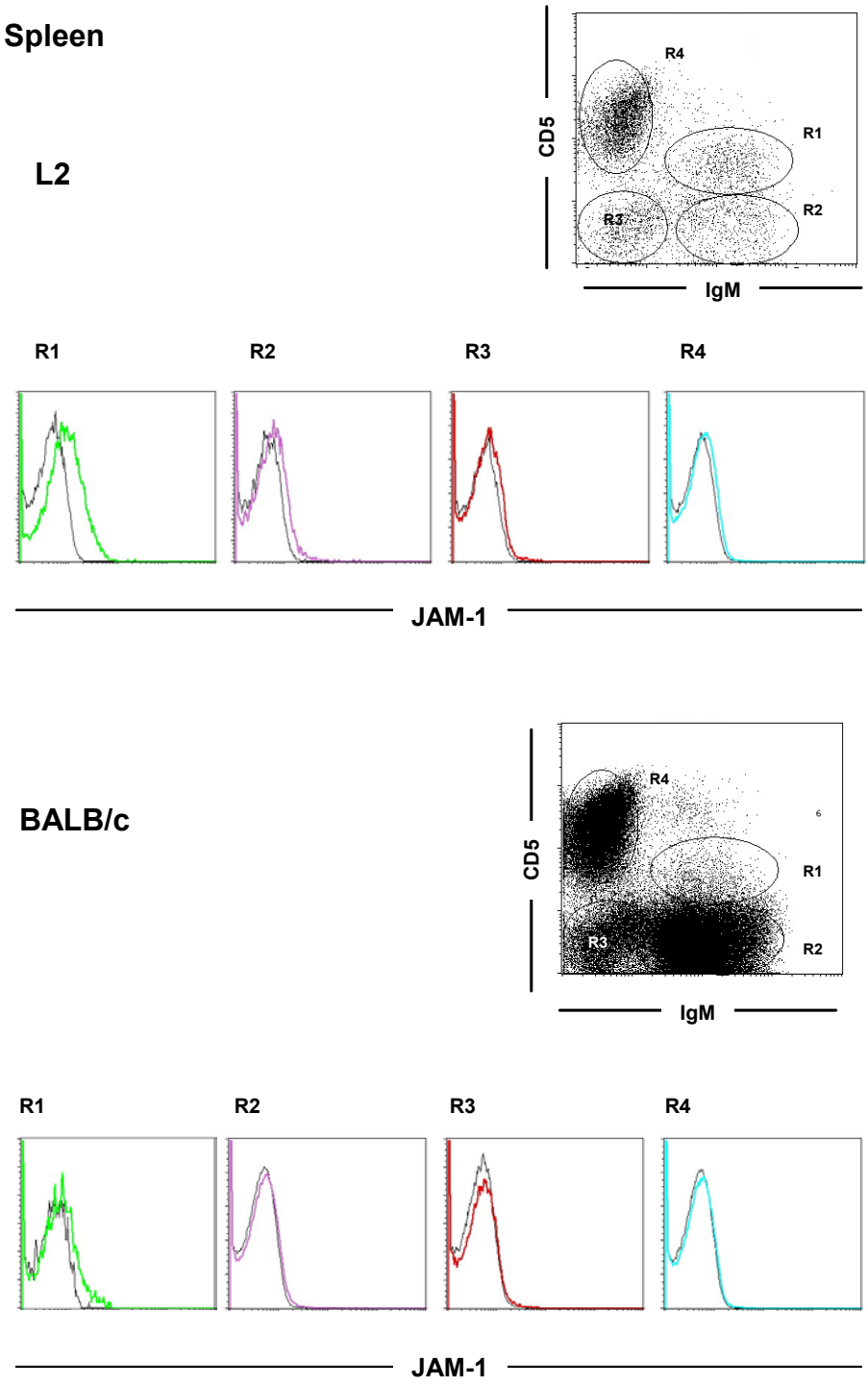


Figure 3.24. Flow cytometrical analysis for JAM-1 expression on spleen cells from L2 and BALB/c mice. Histogram overlays show JAM-1 expression in the following regions: L2 mice: R1: B-1a cells; R2: $CD5^{-}IgM^{+}$ cells containing also marginal zone B cells; R3: double negative cells; R4: T cells. BALB/c: R1: B-1a cells; R2: $CD5^{-}IgM^{+}$ cells containing also marginal zone B cells, R3: $IgM^{lo}CD5^{-}$ containing B-2 cells in BALB/c mice; R4 double negative cells; R5: T cells. Black lines indicate control staining, colored lines represent JAM-1 staining.

3.3 Generation of an anti-idiotype antibody

One of the particular features of L2 mice is that within the population of peritoneal B-1 cells three dominating specificities are found. This was observed by the repetitive finding of heavy chains with identical amino acid sequences even in different individual mice. Such sequences can also be taken as molecular markers for B-1 cells since so far they were not encountered in B-2 cells. Antibodies that would discern such sequences so called anti-idiotypes would represent extremely valuable tools to identify and further characterize such cells in the B-1 cell pool and their physiology.

3.3.1 Hybridoma generation

To be able to generate an anti-idiotype antibody against one of the dominant BCR-specificities, first idiotype bearing antibodies had to be established. Thus, peritoneal and splenic B-1a cells were sorted, stimulated in vitro with LPS and hybridomas were generated by fusion of these cells with the myeloma P3X63Ag8.653.

From the peritoneum 54 hybridomas (designated L2P) and from spleen 21 hybridomas (designated L2M) were obtained and subcloned. cDNA of the heavy chain V regions from these hybridomas was isolated and sequenced to screen for the dominant sequences. Sequences from 27 peritoneum derived and 7 spleen derived hybridomas were obtained (Figure 3.25 and Figure 3.26). The remaining hybridomas either showed no message for IgM in the RT-PCR or ambiguous sequence data were encountered that indicated that more than one hybridoma was present within the sample.

The hybridomas L2P3 and L2M6 displayed V_H sequences that belonged to the most dominating specificity identified before (Figure 3.27). This IgM heavy chain was found in high abundance among the B-1 cells of L2 mice, i.e. 15% of peritoneal, 10% of splenic and 5% of blood B-1a cells as determined by single cell analysis (Kretschmer et al., 2003b). Similar to these studies, different CDR3 region sequences on the nucleotide level were detected in L2P3 and L2M6 (Table 3.3). However, both sequences encoded the same protein. The V region of these antibodies is assembled by gene segments belonging to the V_H Q52, DFL16.2 and J_H4 families and is shown in Figure 3.27.

	CDR3																			V	D	J	-J	V-D	D-J
L2P1	tgt	gca	aga	cac	tat	gat	ggt	tcc	tgg	ttt	gct	tac								7183	SP2	3			N
	cys	ala	arg	his	tyr	asp	gly	ser	trp	phe	ala	tyr													
L2P3	tgt	gcc	aaa	aat	tac	tac	ggc	tac	tac	tat	gct	atg	gac	tac						Q52	FL16.2	4			
	cys	ala	lys	asn	tyr	tyr	gly	tyr	tyr	tyr	ala	met	asp	tyr											
L2P4	tgt	aca	aga	aga	gag	ggg	tat	ggt	aac	cct	cgg	ttt	gct	tac						J558	SP2	3	-1	N	N,P
	cys	thr	arg	arg	glu	gly	tyr	gly	asn	pro	arg	phe	ala	tyr											
L2P7	tgt	gcc	aga	agg	ggt	agt	agc	tac	tct	tat	tac	tat	gct	atg	gac	tac				Q52	FL16.1	4		N	N
	cys	ala	arg	arg	gly	ser	ser	tyr	ser	tyr	tyr	tyr	ala	met	asp	tyr									
L2P13	tgt	acc	agg	cat	tac	tac	ggc	ttt	gac	tac										J606	FL16.2	2	-2	P	
	cys	thr	arg	his	tyr	tyr	gly	phe	asp	tyr															
L2P16	tgt	gcc	aga	aat	tcc	ggg	att	cat	tac	tac	ggc	tac	gac	tac	ttt	gac	tac			Q52	FL16.2	2		N,P	N,P
	cys	ala	arg	asn	ser	gly	ile	his	tyr	tyr	gly	tyr	asp	tyr	phe	asp	tyr								
L2P17	tgt	gcc	aga	ggg	ggg	tgg	cca	acc	ccg	cgg	gag	gct	atg	gac	tac					Q52	n.d.	4	-6	n.d.	n.d.
	cys	ala	arg	gly	gly	trp	pro	thr	pro	arg	glu	ala	met	asp	tyr										
L2P18	tgt	gca	aga	tcg	gta	gta	gtt	gac	tac											J558	FL16.1	2	-4		
	cys	ala	arg	ser	val	val	val	asp	tyr																
L2P19	tgt	gcc	aaa	aat	tgg	ggg	gat	tac	gac	tgt	gct	atg	gac	tac						Q52	SP2.2	4	-5	P,N	N
	cys	ala	lys	asn	trp	gly	asp	tyr	asp	cys	ala	met	asp	tyr											
L2P20	tgt	gcc	aga	ctc	tac	tat	agg	tac	gac	ggg	tac	tat	gct	atg	gac	tac				Q52	SP2.10	4		N	P,N
	cys	ala	arg	leu	tyr	tyr	arg	tyr	asp	gly	tyr	tyr	ala	met	asp	tyr									
L2P22	tgt	gcc	aga	gtc	tac	tat	agg	tac	gac	gga	tac	tat	gct	atg	gac	tac				Q52	SP2.10	4			N,P
	cys	ala	arg	val	tyr	tyr	arg	tyr	asp	gly	tyr	tyr	ala	met	asp	tyr									
L2P23	tgt	gca	aga	tcg	ggt	att	act	acg	gta	gta	gct	caa	tat	tac	tat	gct	atg	gac	tac	7183	FL16.1	4		N,P	N
	cys	ala	arg	ser	gly	ile	thr	thr	val	val	ala	gln	tyr	tyr	tyr	ala	met	asp	tyr						
L2P25	tgt	gca	aga	cac	gat	gga	aac	tgg	gac	tac	ttt	gac	tac							J558	DQ52	2		N,P	
	cys	ala	arg	his	asp	gly	asn	trp	asp	tyr	phe	asp	tyr												
L2P28	tgt	gca	aga	gac	tac	ggt	agt	agc	tac	tat	gct	atg	gac	tac						J558	FL16.1	4			
	cys	ala	arg	asp	tyr	gly	ser	ser	tyr	tyr	ala	met	asp	tyr											
L2P29	tgt	gca	aga	cac	gaa	gct	aac	tgg	gac	tac	ttt	gac	tac							VNP	Q52	2			
	cys	ala	arg	his	glu	ala	asn	trp	asp	tyr	phe	asp	tyr												
L2P30	tgt	gca	aga	ttt	act	acg	gta	gta	gct	gac	tac									J558	FL16.1	2	-5		
	cys	ala	arg	phe	thr	thr	val	val	ala	asp	tyr														
L2P31	tgt	gcg	aga	gac	ttc	gat	gtc													GAM3-8	n.d.	1	-7	n.d.	n.d.
	cys	ala	arg	asp	phe	asp	val																		
L2P33	tgt	aca	aga	ttc	tac	tac	ggt	agt	agc	tac	tac	ttt	gac	tac						J558	FL16.1	2		N	
	cys	thr	arg	phe	tyr	tyr	gly	ser	ser	tyr	tyr	phe	asp	tyr											
L2P34	tgt	gcc	aga	aag	cat	ggt	aac	tac	gtt	gac										Q52	SP2	2	-4	P	P
	cys	ala	arg	lys	his	gly	asn	tyr	val	asp															
L2P36	tgt	gca	aga	act	ggg	acg	ttt	gac	tac											J558	Q52	2	-3		P
	cys	ala	arg	thr	gly	thr	phe	asp	tyr																
L2P37	tgt	gca	aga	gct	gac	gtt	tat	gct	atg	gac	tac									GAM3-8	n.d.	4	-3	n.d.	n.d.
	cys	ala	arg	ala	asp	val	tyr	ala	met	asp	tyr														
L2P38	tgt	gcc	aaa	aac	ccc	ctc	att	act	acg	gca	cgg	ggg	tac	tat	gct	atg	gac	tac		Q52	FL16.2	4		N	N
	cys	ala	lys	asn	pro	leu	ile	thr	thr	ala	arg	gly	tyr	tyr	ala	met	asp	tyr							
L2P40	tgt	gcc	aga	ctc	tac	tat	agg	tac	gac	ggg	tac	tat	gct	atg	gac	tac				Q52	SP2.10	4		N	N,P
	cys	ala	arg	leu	tyr	tyr	arg	tyr	asp	gly	tyr	tyr	ala	met	asp	tyr									
L2P41	tgt	gcc	aga	gat	aga	ggt	tac	tac	ggt	agt	agc	tat	tac	gct	atg	gac	tac			Q52	FL16.1	4		N	P
	cys	ala	arg	asp	arg	gly	tyr	tyr	gly	ser	ser	tyr	tyr	ala	met	asp	tyr								
L2P42	tgt	gca	agc	ttc	tat	ggt	tac	tat	gct	atg	gac	tac								J558	SP2	4		P,N	N
	cys	ala	ser	phe	tyr	gly	tyr	tyr	ala	met	asp	tyr													
L2P43	tgt	gcc	aga	gat	acc	tac	tat	agg	tcc	ttt	gac	tac								Q52	SP2	2	-2	N	N
	cys	ala	arg	asp	thr	tyr	tyr	arg	ser	phe	asp	tyr													
L2P46	tgt	gca	agc	cgg	gac	ggc	ttt	gct	tac											J558	FL16.2	3	-3	P,N	
	cys	ala	ser	arg	asp	gly	phe	ala	tyr																

Figure 3.25. CDR3 sequences from hybridomas generated from peritoneal B-1a cells of L2 mice. The CDR3 nucleotide and amino acid sequence is displayed starting with Cys in position 92 of the V_H 3' region and including the D gene segment and the J_H 5' region up to position 102. The V_H gene family, the D segment and the J_H segment are indicated (VDJ). In some cases no D segment could be determined (n.d.). Numbers in the (-J) column indicate the number of nucleotides that have been removed from J sequence due to nibbling. P and N indicate the presence of P-nucleotides and N-nucleotides in the V-D and D-J junctions of the respective sequences.

	CDR3																	V	D	J	-J	V-D	D-J
L2M6	tgt	gcc	aaa	aat	tac	tac	ggc	tat	tac	tat	gct	atg	gac	tac									
	cys	ala	lys	asn	tyr	tyr	gly	tyr	tyr	tyr	ala	met	asp	tyr				Q52	FL16.2	4			P
L2M8	tgt	gcc	aga	aat	ggg	agg	act	acg	gcg	tac	tat	gct	atg	gac	tac								
	cys	ala	arg	asn	gly	arg	thr	thr	ala	tyr	tyr	ala	met	asp	tyr			Q52	FL16.2	4		N	P
L2M12	tgt	gca	aga	cac	gaa	gta	ggc	cgg	ctc	ctc	att	act	acg	gcc	ccg	ttt	gct	tac						
	cys	ala	arg	his	glu	val	gly	arg	leu	leu	ile	thr	thr	ala	pro	phe	ala	tyr	J558	FL16.2	3	-3	N	N
L2M14	tgt	gca	aga	tac	tac	ggc	gac	tac	ttt	gac	tac													
	cys	ala	arg	tyr	tyr	gly	asp	tyr	phe	asp	tyr								J558	FL16.2	2		P	P,N
L2M15	tgt	gcc	aga	ctc	tac	tat	agg	tac	gac	ggg	tac	tat	gct	atg	gac	tac								
	cys	ala	arg	leu	tyr	tyr	arg	tyr	asp	gly	tyr	tyr	ala	met	asp	tyr			Q52	SP2.10	4		N	P,N
L2M16	tgt	gtg	agc	tac	tat	gat	tac	gcc	tgg	ttt	gct	tta												
	cys	val	ser	tyr	tyr	asp	tyr	ala	trp	phe	ala	tyr							DNA4	SP2.2	3			P
L2M17	tgt	gca	aga	gag	gat	ttt	act	acg	gct	acg	gac	tac												
	cys	ala	arg	glu	asp	phe	thr	thr	ala	thr	asp	tyr							J558	FL16.2	2	-6	N	P

Figure 3.26. CDR3 sequences of hybridomas from splenic B-1a cells of L2 mice. For further explanations see Figure 3.25.

A second sequence was obtained twice, once from a peritoneal hybridoma (L2P40) and second from a splenic hybridoma (L2M15) (Figure 3.28). Both sequences were identical on nucleotide and protein level. This sequence had also been found before and made up 5% of the sequences found in the blood of L2 mice (Kretschmer et al., 2003b). It was not found in spleen or peritoneum before. The V region of this antibody consists of rearranged family members of V_H Q52, DSP and J_H4 gene segments.

Table 3.3. Comparison of CDR3 regions of L2P3 and L2M6. Differences in nucleotide sequence are highlighted in green.

	CDR3													
L2P3	tgt	gcc	aaa	aat	tac	tac	ggc	tac	tac	tat	gct	atg	gac	tac
L2M6	tgt	gcc	aaa	aat	tac	tac	ggc	tat	tac	tat	gct	atg	gac	tac
Protein	**cys**	**ala**	**lys**	**asn**	**tyr**	**tyr**	**gly**	**tyr**	**tyr**	**tyr**	**ala**	**met**	**asp**	**tyr**

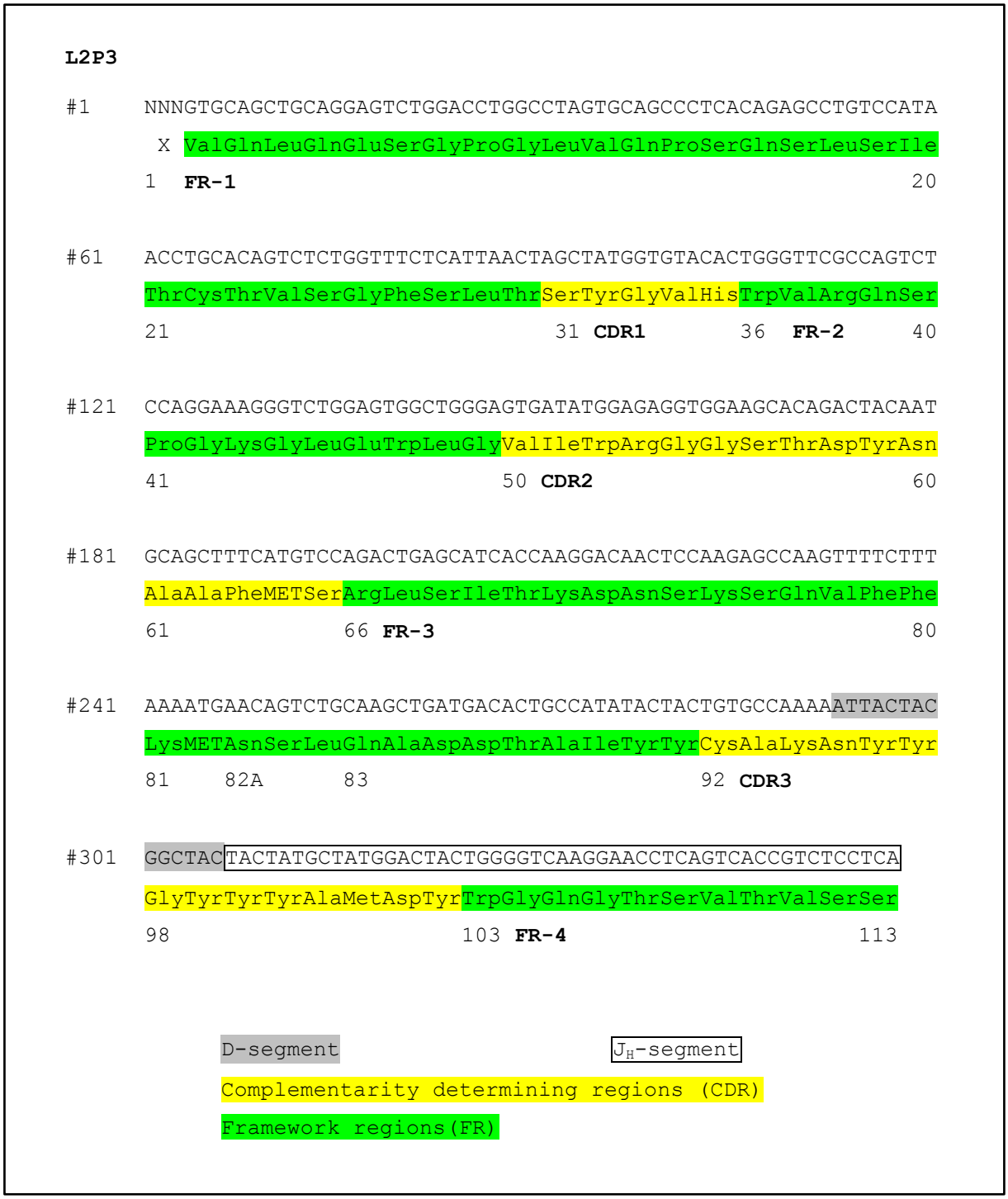


Figure 3.27. V-region sequence of L2P3. The nucleotide and amino acid (aa) sequences are shown. N indicates nucleotides that could not be determined due to ambiguities in sequence data. The resulting gaps on aa level are indicated by an X. CDR1 and CDR2 as well as the frame work regions were determined according to Kabat et al. (Kabat et al., 1987) and are highlighted as explained in the legend. The CDR3 region is displayed as the region including the conserved cysteine residue in position 92 of the V_H and the tyrosine in position 102 of the J_H. Thus, in contrast to the commonly used CDR3 definition three additional aa of the V_H are included according to the definition used in preceding work (Kretschmer et al., 2002; Kretschmer et al., 2003b).

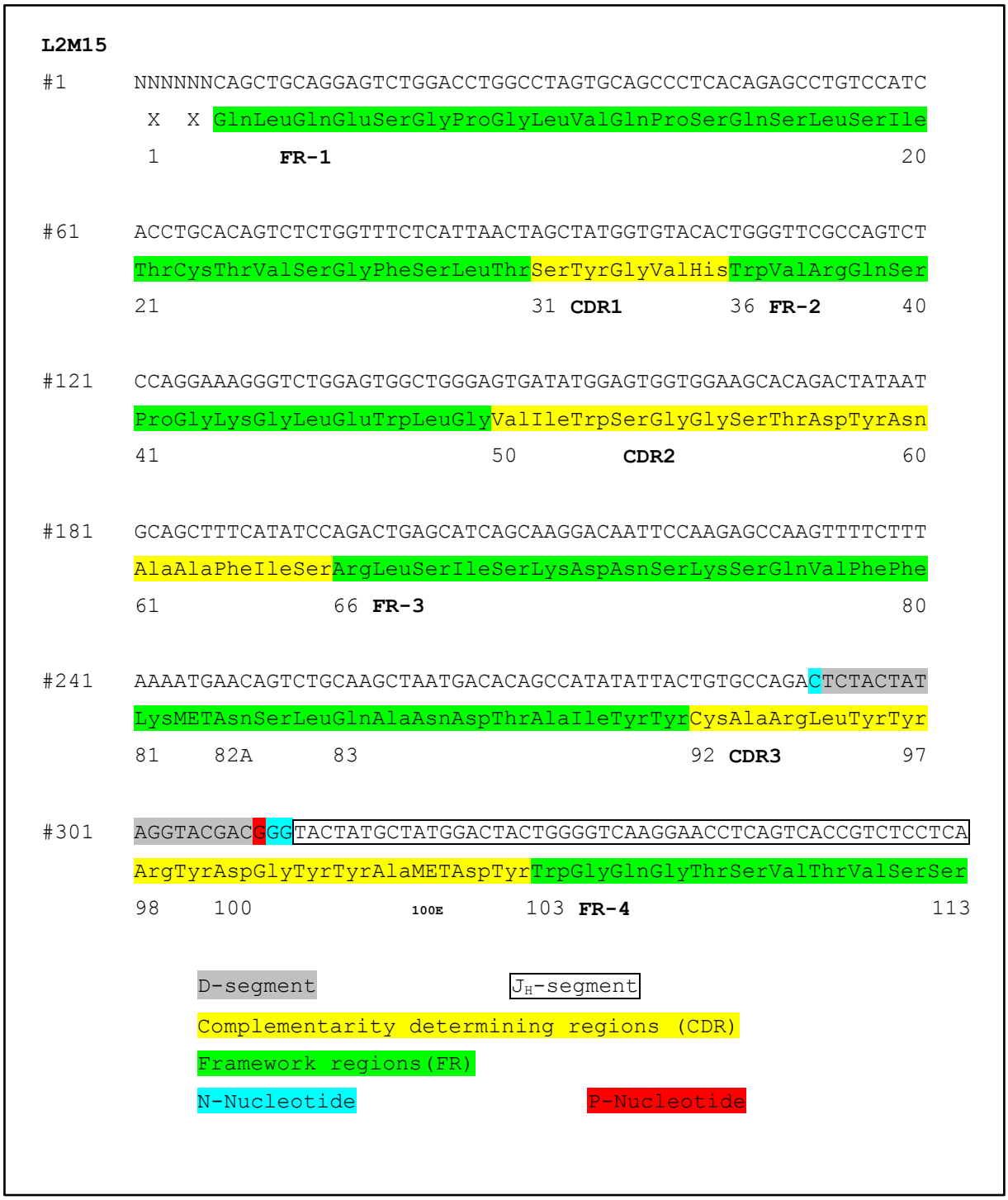


Figure 3.28. V-region sequence of L2M15. The sequence is identical with the sequence obtained from L2P40. For further explanations see Figure 3.27.

The antibodies of L2P3 and L2M15 as well as antibodies secreted by the plasmacytoma MOPC104E (IgM/λ1) were purified from cell culture supernatants by ammonium sulfate precipitation and affinity chromatography using immobilized mannan binding protein (ImmunoPure). L2P3 was then used as an antigen to

immunize rats and to generate hybridomas by fusion of spleen cells with P3X63Ag8.653 cells. This work was done in cooperation with the lab of Dr. Elisabeth Kremmer at the GSF in Munich. Supernatants of the rat hybridomas were screened in ELISA for binding to purified antibodies of L2P3, MOPC104E (IgM/λ) as well as an IgM/κ antibody. Thus, one could exclude clones producing antibodies against the IgM heavy chain, κ or λ light chains. As the antibody from the hybridoma L2M15 (IgM/λ) is very similar to L2P3 (Figure 3.29) including usage of V_HQ52 and J_H4 gene segments it was included in the ELISA screenings as control for the anti-idiotypic reactivity.

One clone was identified that produced antibodies that bound only the L2P3 antibody when tested in ELISA. This idiotype specific antibody was named 8H10 according to the clone number. Supernatants of this clone were used for further analyses.

```
          1                                                    20
L2P3      XXXValGlnLeuGlnGluSerGlyProGlyLeuValGlnProSerGlnSerLeuSerIle
L2M15     XXXXXX -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -


          21                                                   40
L2P3      ThrCysThrValSerGlyPheSerLeuThrSerTyrGlyValHisTrpValArgGlnSer
L2M15      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          41                                                   60
L2P3      ProGlyLysGlyLeuGluTrpLeuGlyValIleTrpArgGlyGlySerThrAspTyrAsn
L2M15      -  -  -  -  -  -  -  -  -  -  -  - Ser -  -  -  -  -  -  -  -

          61                                                   80
L2P3      AlaAlaPheMETSerArgLeuSerIleThrLysAspAsnSerLysSerGlnValPhePhe
L2M15      -  -  - Ile -  -  -  -  - Ser -  -  -  -  -  -  -  -  -  -  -

          81    82A   82C                                      97
L2P3      LysMETAsnSerLeuGlnAlaAspAspThrAlaIleTyrTyrCysAlaLysAsnTyrTyr
L2M15      -  -  -  -  -  -  - Asn -  -  -  -  -  -  -  - ArgLeu -  -

          98
L2P3      GlyTyr ·  · TyrTyrAlaMETAspTyrTrpGlyGlnGlyThrSerValThrValSer
L2M15     Arg - AspGly -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

          113
L2P3      Ser
L2M15      -
```

Figure 3.29. Comparison of L2P3 and L2M15 protein sequence. Dashes indicate matching amino acids, dots indicate gaps necessary for alignment. Triple X indicates amino acids that could not be determined due to ambiguities in the nucleotide sequence.

Flow cytometrical analyses were performed using the supernatants from the rat hybridoma 8H10. First, peritoneal B-1 cells from L2 mice were tested for binding to the anti-idiotype antibody (data not shown). As secondary reagent a fluorochrome coupled $F(ab')_2$ fragment of a mouse anti-rat IgG (H+L) was used which produces some staining background. Staining of peritoneal cells of L2 mice could indeed be observed. Peritoneal cells from L2 mice were then used to optimize the 8H10 staining for flow cytometry (data not shown).

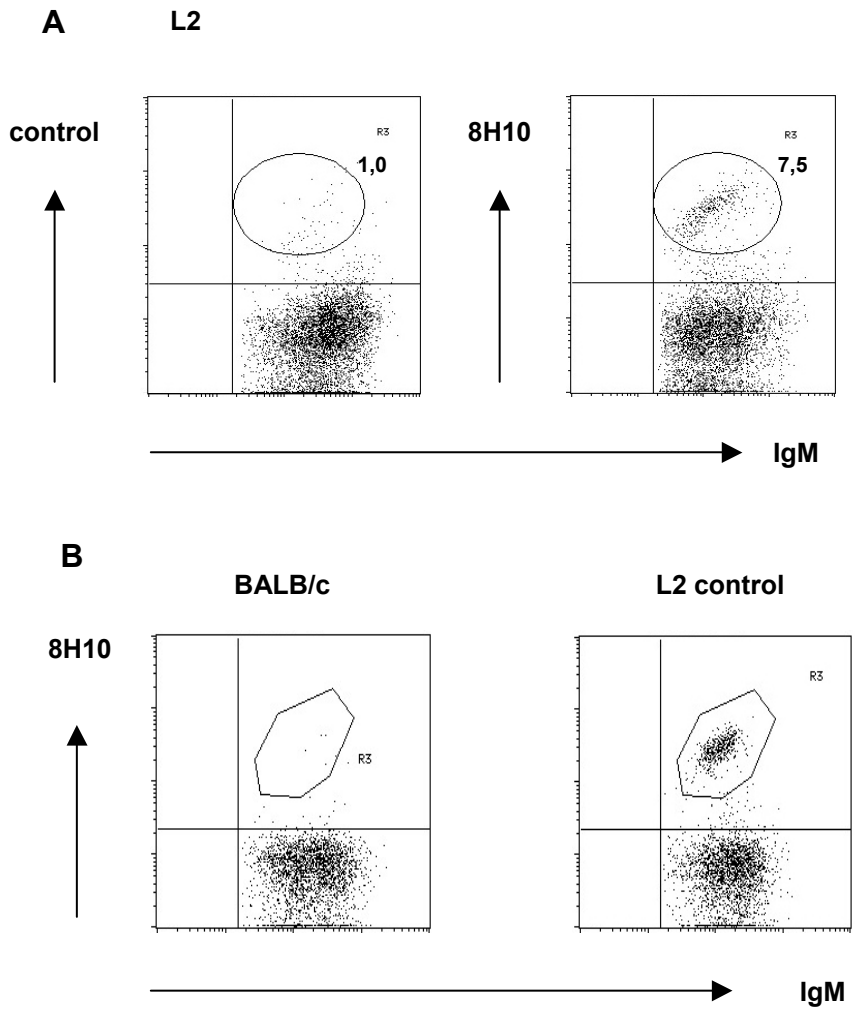


Figure 3.30. 8H10 staining on peritoneal cavity cells. A) Staining of pooled peritoneal cavity cells from L2 mice. Background by secondary staining reagent is indicated in the left dot plot. Subtraction of background staining of 8H10 stained cells reveals a 6,5% population of $8H10^+$ cells. Pooled cells were obtained from 2 male and 1 female L2 mice. B) Staining of peritoneal cells from female BALB/c mice. Dotplot from one BALB/c mouse is representative for three individual mice tested. Peritoneal cavity cells of one L2 mouse were included as positive control.

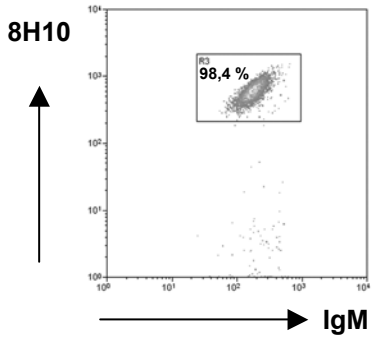
Initial stainings using the detection system described above showed a 6,5% population (background subtracted) of $8H10^+IgM^+$ double positive cells on pooled peritoneal cavity cells from L2 mice (Figure 3.30A). This is consistent with an anti-idiotype that recognizes a major specificity in transgenic L2 mice. However, peritoneal cells from BALB/c mice were not positive for 8H10 stainings (Figure 3.30B). Therefore, further analyses were focused on L2 mice.

3.3.2 Analysis of 8H10 specificity and flow cytometrical analysis

Next, the specificity of the 8H10 antibody was analyzed. Therefore, $8H10^+IgM^+$ double positive peritoneal cells of L2 mice were sorted. cDNA of IgM heavy chains was prepared and amplified by PCR with primers specific for the V region. Then the PCR products were subcloned and individual V-region sequences determined. It was not possible to reveal the complete V-region with the obtained sequence data. However, CDR3 sequences of all clones could be analyzed. 23 of 34 sequences showed the same CDR3 region as L2P3 (Figure 3.31). 19 of these were completely identical with L2P3 as far as the sequences could be compared. The remaining four sequences showed single nucleotide exchanges, that lead to an exchange of amino acids. These single changes could be indicative for somatic hypermutation but could also be due to errors of the Taq-polymerase used for RT-PCR. However, their CDR3 region matched with the L2P3 CDR3. Four sequences of the 34 analyzed sequences differed in their CDR3 regions from that of L2P3 (Figure 3.31 B and C, #37, 40, 44, 45). Of the seven remaining sequences analyzed none encoded a functional heavy chain. Most likely these sequences are derived from rearrangements that did not yield a correct reading frame. Therefore, these sequences are not translated into protein. Thus, of 34 sequences analyzed only 27 encode a functional BCR. Of these, 23 were identical with L2P3 representing 85% of the functional sequences.

The analysis of idiotype bearing cells from L2 mice was extended to single L2 mice. On average 6% of IgM^+ cells were detected by the $8H10^+$ antibody in the peritoneal cavities in male L2 mice (Figure 3.32) which confirmed the data obtained by analysis of pooled cells before. However, this result differed from the original expectations since 15% the L2P3 idiotype were predicted for the peritoneal cavity using single cell RT-PCR and results obtained from analyses of hybridoma sequences (Kretschmer et al., 2002; Kretschmer et al., 2003b).

A



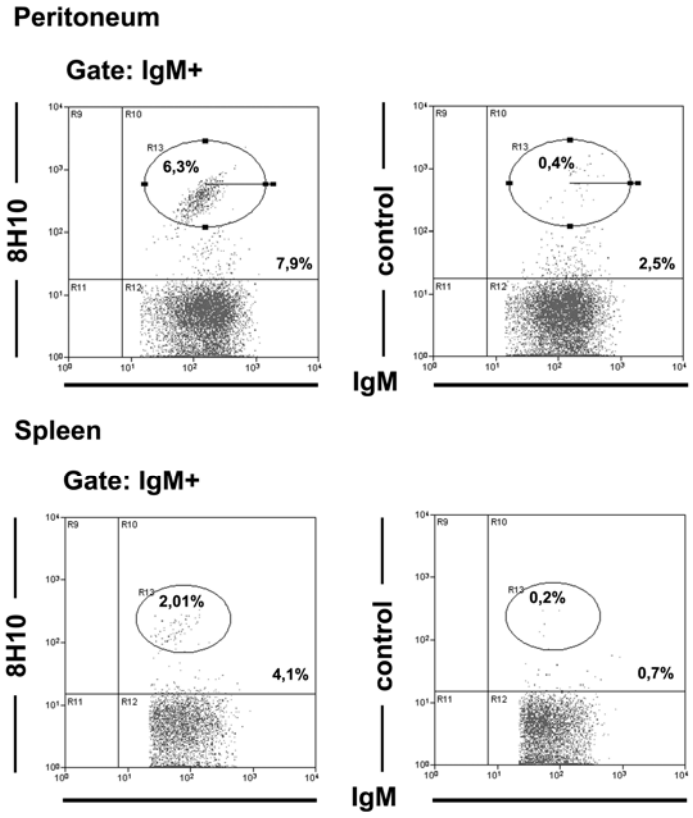
B

Designation	Sequence readable from amino acid position	Comparison with L2P3 protein sequence
13	27	nf
14	25	-
15	30	nf
16	32	-
17	44	nf
18	25	-
19	33	-
20	43	-
21	25	-
22	28	-
23	26	-
24	23	-
25	19	Ser 19→ Cys
26	49	nf
27	12	Ser 19 → Cys
28	43	-
29	30	-
31	44	nf
32	49	-
34	25	Gly 44 → Phe
35	49	-
36	49	-
37	26	CDR3 differs from P3
38	26	-
39	14	nf
40	26	CDR3 differs from P3
41	15	-
42	28	nf
43	26	Val 34→ Phe
44	18	CDR3 differs from P3
45	15	CDR3 differs from P3
46	14	-
47	15	-
48	30	-

C

	CDR3																		V	D	J
L2P3	tgt	gcc	aaa	aat	tac	tac	ggc	tac	tac	tat	gct	atg	gac	tac					Q52	FL16.2	4
	cys	ala	lys	asn	tyr	tyr	gly	tyr	tyr	tyr	ala	met	asp	tyr							
37	tgt	gcc	aga	aat	ttc	cca	ccg	cat	tac	tat	gct	atg	gac	tac					Q52	FL16.2	4
	-	-	arg	-	phe	pro	pro	his	-	-	-	-	-	-							
40	tgt	gcc	aga	aat	cgt	ccc	tta	tat	tac	tat	gct	atg	gac	tac					Q52	FL16.1	4
	-	-	arg	-	arg	pro	leu	tyr	-	-	-	-	-	-							
44	tgt	gca	aga	cat	gaa	ctt	gac	tac											7183	n.d.	2
	-	-	arg	his	glu	leu	asp	tyr													
45	tgt	gcc	aga	gat	tat	agg			tac	tat	gct	atg	gac	tac					Q52	SP2.10	4
	-	-	arg	asp	-	arg			-	-	-	-	-	-							

Figure 3.31. Sequences of sorted peritoneal 8H10$^+$IgM$^+$ cells from L2 mice. A) Reanalysis of 8H10$^+$IgM$^+$ sorted cells. B) Summary of sequence data. The designation of the individual sequences is given and the amino acid (aa) position from which sequence data could be analyzed and compared with L2P3 sequence. Dash indicates identical aa sequence with L2P3; nf: no functional reading frame assignable. Ser 19 → Cys indicates that serine found at position 19 in L2P3 is exchanged by cysteine in the compared sequence. C) CDR3 sequences from clones that differed from L2P3 CDR3 sequence. Amino acids matching with L2P3 CDR3 aa are indicated by dashes; n.d. indicates that no D segment was assignable.



	% of 8H10 positive cells on gated IgM+ cells [background substracted]	
L2 individuals	Peritoneum	Spleen
1	9,2	2,0
2	5,9	1,8
3	3,9	0,9
4	4,4	1,4
5	7,4	1,3
Mean expression	**6,1 ± 2,2**	**1,5 ± 0,5**

Figure 3.32. Analysis of 8H10 stainings performed on cells from spleen and peritoneal cavity of male L2 mice. Dotplots represent stainings of one mouse. The table summarizes staining results of individual mice. Background by detection antibody is subtracted in the table.

Similarly, in the spleens of these mice also lower amounts of $8H10^+$ IgM^+ cells were detected than expected from single cell analysis. Results obtained by this method predicted 10% of $8H10^+$ IgM^+ cells in the spleen whereas only 1,3% on average could be detected in the flow cytometrical stainings from individual mice.

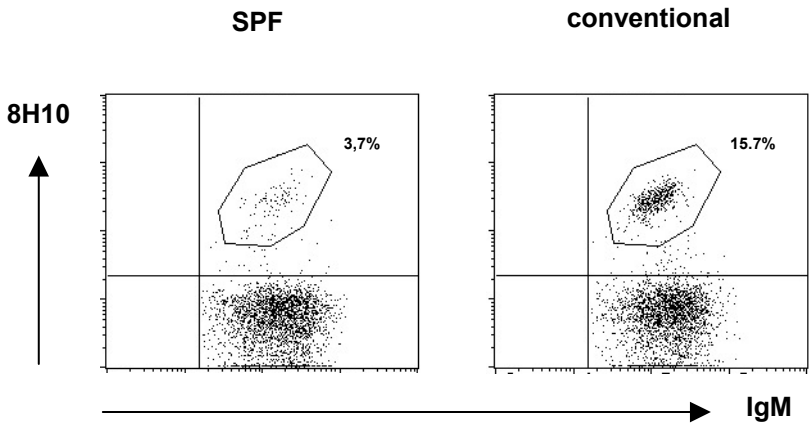
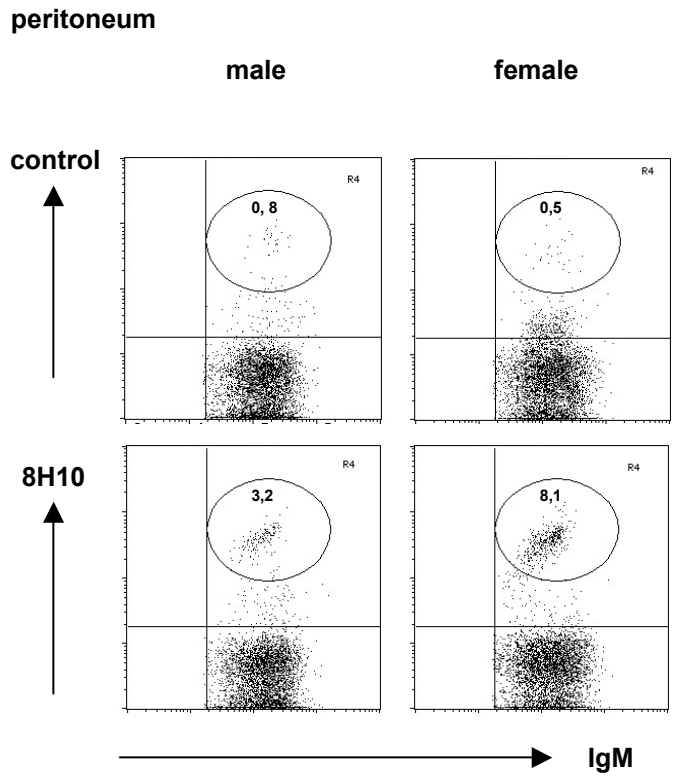


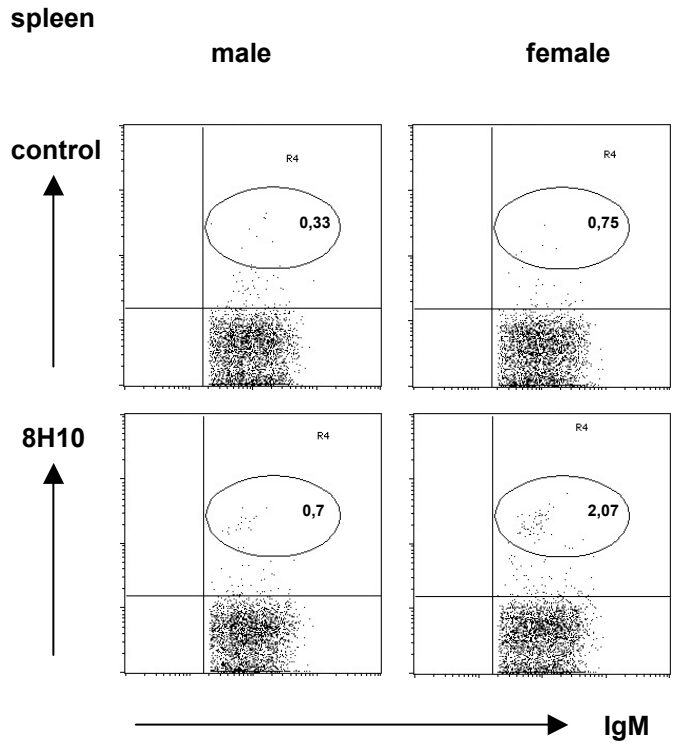
Figure 3.33. Flow cytometrical analysis of peritoneal cells from female mice raised under SPF and conventional conditions. The staining was performed on pooled peritoneal cells from four female mice from SPF conditions and one female mouse obtained from our normal mouse colony.

Interestingly, when pooled peritoneal cells from female L2 mice bred under SPF conditions were analyzed, such mice showed a low percentage of $8H10^+$ cells (Figure 3.33). A female mouse that was housed under conventional conditions and was included as a control showed 15% $8H10^+$ cells, the number expected from single cell RT-PCR. As mainly male mice were used in the preceding stainings a difference in idiotype expression due to the gender of the mouse was possible. This idea was supported by the notion that the single cell studies were also performed with cells from female mice. Therefore, the stainings using the 8H10 antibody were repeated in male and female L2 mice (Figure 3.34).

The percentage of $8H10^+$ cells detected in the peritoneal cavity was lower in male mice compared to the stainings in Figure 3.32. Similarly, in spleens lower percentages of these cells were found. Female mice in contrast showed a higher percentage of $8H10^+$ cells in the peritoneal cavity than male mice. In the spleen a similar finding was observed. There was an average 1,3% population of $8H10^+$ cells detectable in female mice whereas in male mice 0,4% of $8H10^+$ cells where found. These results indicate, that there could be an impact of gender on the expression of the L2P3 specificity. However, also the hygienic conditions might play an important role as indicated by the differences observed between mice housed under conventional and SPF (specific pathogen free) conditions.



	[%] of 8H10 on IgM+ gated cells	
#	male	female
1	2,4	7,6
2	3,1	12,5
3	4,4	4,8
mean	3,42 ± 1,2	8,3 ± 3,9



	[%] of 8H10 on IgM+ gated cells	
#	male	female
1	0,4	1,1
2	0,1	1,7
3	0,7	1,3
mean	0,4 ± 0,3	1,3 ± 0,3

Figure 3.34. Comparison of anti-idiotype stainings on L2 mice. Dotplots show representative stainings of a female and a male L2 mouse. A) Staining of peritoneal cells. Background by secondary antibody is subtracted in the table. B) Staining of splenic cells. Table summarizes results from three individual mice. Significance of gender difference was determined using the one sided Wilcoxon's two sample test. Peritoneum: $p < 0,05$, Spleen: $p<0,01$.

3.4 Analysis of L2P3 binding

The B-1 cell derived antibody pool is highly enriched for autoreactive specificities that bind to intra- and extracellular autoantigens like single-stranded DNA or carbohydrate epitopes of glycolipids and –proteins (Baumgarth et al., 2005). For instance the work of Hayakawa and colleagues demonstrated the important role of autoantigen for the selection and maintenance of B-1 cells (Hayakawa et al., 1999). They used a transgenic mouse expressing a B-1 cell derived anti-Thy-1 IgM antibody (ATA; V_H3609-D_HQ52-J_H2, V_κ21C-J_κ2). The presence of the Thy-1 autoantigen yielded an increased frequency of $CD5^+$ transgenic B-1 cells. They also observed an age-dependent accumulation of $CD5^+$ transgenic B cells suggesting cellular selection even after the generation of these cells.

Similarly, the clones dominating in the B-1 cell pool of L2 mice might be the result of autoantigen-dependent selection mechanisms (Kretschmer et al., 2002). Thus, L2P3, representing one of the dominating specificities and L2M15, that was also repeatedly found in blood of L2 mice were tested for their potential to detect autoantigens in L2 mice. As a first and simple approach L2P3 and L2M15 were tested in flow cytometrical analysis using cells from various organs of CB20/L2 mice. These mice are congenic BALB/c mice that carry the immunoglobulin locus of C57BL/6 and therefore express the IgM^b allotype. Since L2P3 and L2M15 antibodies are derived from BALB/c L2 mice the IgM^a allotype could be distinguished from endogenous IgM^b by a fluorochrome coupled anti-IgM^a detection antibody. Therefore, cells from peritoneum, spleen, bone marrow and thymus were isolated and stained with L2P3 and L2M15, respectively. MOPC104E was included as a possible negative control.

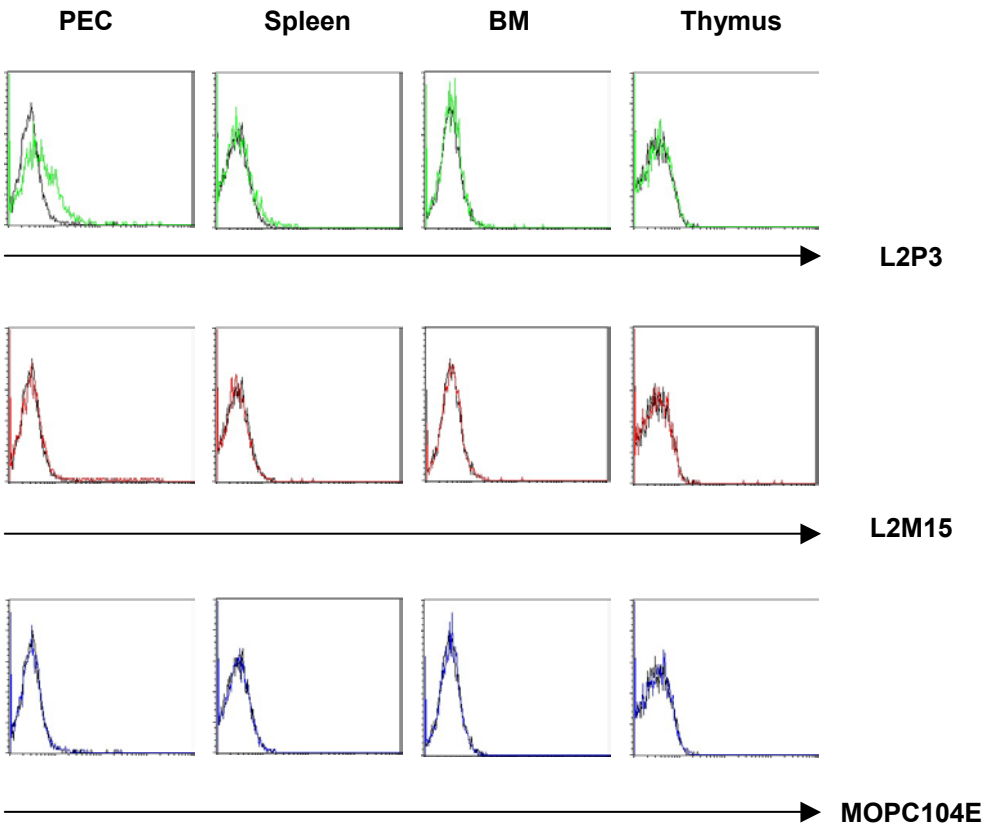


Figure 3.35. Flow cytometrical studies of L2P3 and L2M15 binding. Cells derived from the peritoneum (PEC), spleen, bone marrow (BM) and thymus of CB20/L2 mice were gated on lymphoid cells in flow cytometry and stained with L2P3, L2M15 and the control IgM/λ antibody MOPC104E. Samples consisted of pooled cells obtained from the indicated anatomical sites of two female and one male CB20/L2 mouse. Black line represents controls stained with the IgM^a detection antibody omitting the primary antibody. Colored lines represent staining with the antibodies L2P3, L2M15 and MOPC104E as indicated.

As seen in the histograms of Figure 3.35, L2P3 generated a shift in staining intensity in the lymphoid cell population derived from the peritoneal cavity but not on cells obtained from spleen, bone marrow or thymus. The second B-1a cell derived antibody, L2M15, as well as the control antibody MOPC104E, however, showed no reactivity with any of the tested populations. Thus, the staining obtained with L2P3 indicates a specific staining. Therefore, L2P3 might detect an autoantigenic determinant exposed on lymphoid cells in the peritoneal cavity.

The staining was repeated with a second pool of cells obtained from the peritoneal cavity or the spleen of CB20/L2 mice and cells in the lymphoid gate were analyzed in flow cytometry. A similar shift inflicted by L2P3 (Figure 3.36) was observed in this experiment. No shift was seen with splenic cells stained using this antibody. The

stainings with L2M15 and MOPC104E were negative. Thus the binding of L2P3 observed on cells found within in the peritoneal lymphoid gate was confirmed.

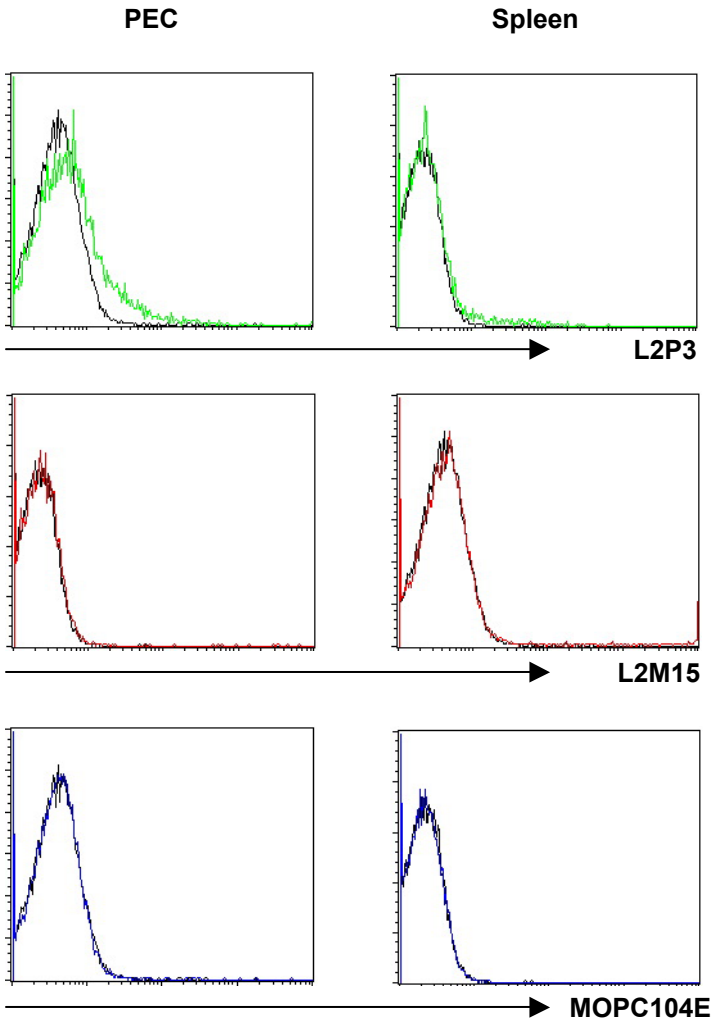


Figure 3.36. Flow cytometrical studies of L2P3 and L2M15 binding. Cells derived from the peritoneum (PEC) and spleen of CB20/L2 mice were gated on lymphoid cells in flow cytometry and stained with L2P3, L2M15 and the control IgM/λ antibody MOPC104E. Samples consisted of pooled cells obtained from the indicated anatomical sites of three male CB20/L2 mice.

Surprisingly, when non-transgenic mice were tested no staining with L2P3 was observed (data not shown). This seems to indicate that for unknown reasons this autoantigen is found only in the transgenic situation on lymphoid cells of the peritoneal cavity.

3.4.1 Half-lives of B-1 cell derived antibodies

The fact that B-1 cell derived antibodies might recognize autoantigens should be reflected in their *in vivo* persistence, i.e. such antibodies should exhibit a short half life compared to B-2 cell derived antibodies. Therefore L2P3 and L2M15 were injected intravenously into CB20/L2 mice and sera from these mice were collected at different time points to determine the rate of clearance of such antibodies from the sera. As controls two additional antibodies, MOPC104E (IgM, λ1) and 2D4 (IgM, κ), were included in these studies. MOPC104E is derived from a plasmacytoma that was induced in BALB/c mice by intraperitoneal injection of mineral oil. This myeloma might have arisen from B-1 cells. 2D4 is a hybridoma the antibody of which is directed against ganglio-N-triosylceramide (asialo GM_2) (Young, Jr. et al., 1979) and was obtained after immunization, thus, should be of B-2 origin although autoreactivity can not be excluded for the moment. Serum samples of individual mice were obtained at variant time points after injection and submitted to a IgM^a specific ELISA. The optical density value at the first experimental time point was arbitrarily set as 100% and the following values correlated to it.

Interestingly, L2P3, L2M15 and MOPC104E showed two rates of clearance (Figure 3.37A). The first phase showed a rapid decrease of the antibodies in sera, the second phase displayed a slower clearance. In contrast, the clearance rate of 2D4 from sera did not seem to underlie a biphasic mechanism. It could be described by a trendline based on an exponential function (Figure 3.37B) which is represented by a straight line in a semi-logarithmic diagram. Therefore the kinetic of 2D4 showed only one phase. The results showed that all B-1 derived antibodies behaved similar, i.e. in the first phase the half life ($t_{1/2}$) was around 10 hours and in the second phase around 35-40 hours while $t_{1/2}$ of the B-2 derived antibody was 22 hours. This might be due to the different nature of the antigens recognized by the B-1 versus B-2 cell derived antibodies, i.e. reaction with an autoantigen or not.

Together the studies using antibodies of B-1 cells from L2 mice added several important aspects to the study of B-1 cells and their antibodies. The generation of the 8H10 anti-idiotype antibody extends the results obtained by sequencing so far and will in future allow simplified experiments on the repertoire and selection of these cells.

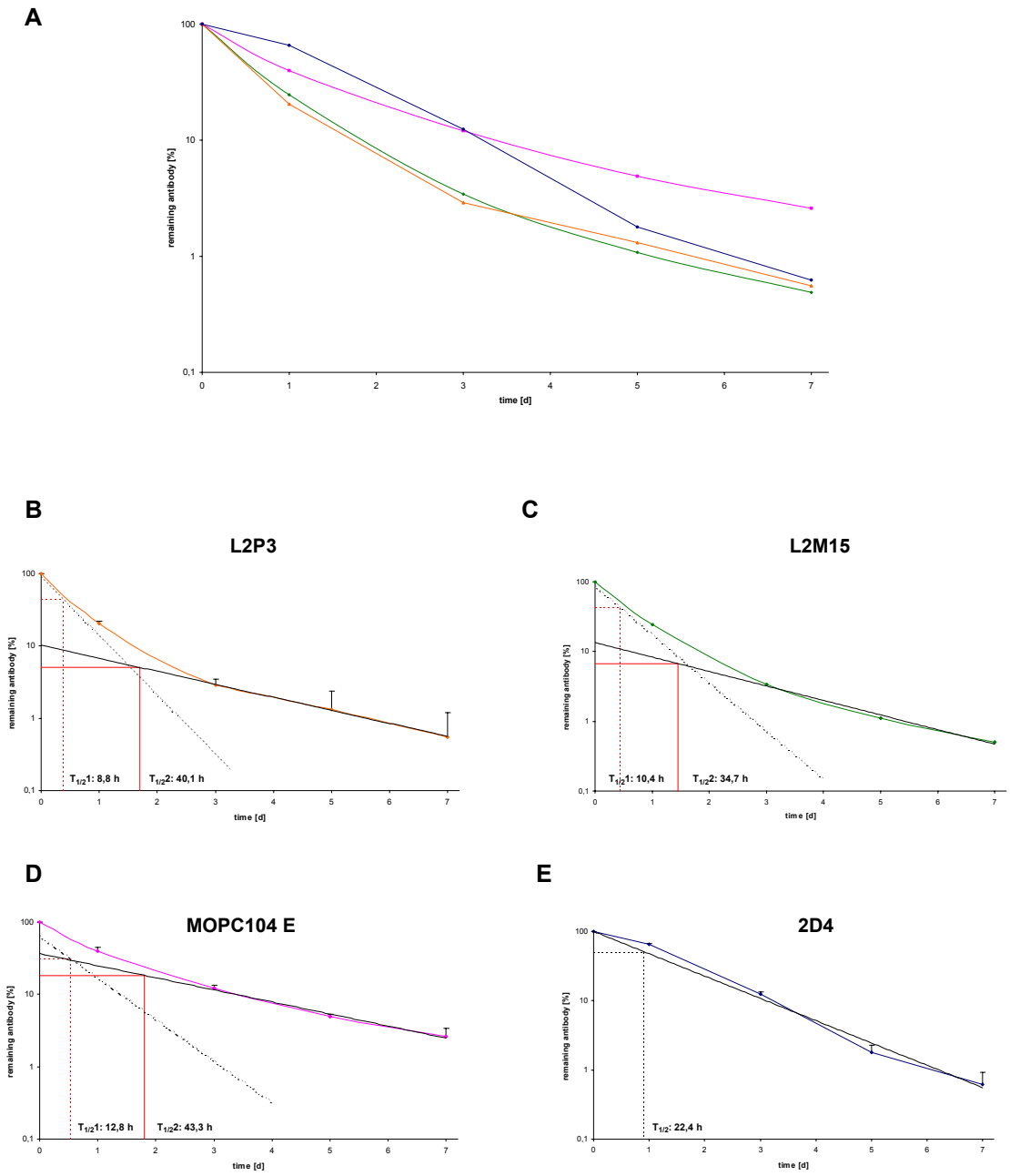


Figure 3.37 Clearance of L2P3, L2M15, MOPC104E and 2D4 of sera from CB20/L2 mice. A) Clearance curves for L2P3 (orange), L2M15 (green) and MOPC104E (pink) and 2D4 (grey) are displayed. B-E) Graphical evaluation the kinetics of the indicated antibodies. Trendlines for the first and second phases are indicated by black dashed or black solid lines, respectively. Red dashed and solid lines indicate the determination of antibody half-life from the diagrams. Half-life of the antibodies in the two phases are shown in the diagrams ($T_{1/2}$). Data points for all curves represent mean values from three individual mice except the curve for L2M15 which is based on data of two mice.

4 Discussion

In the present work novel features of murine B-1 cells were studied in order to better characterize physiological peculiarities of this B cell population. To this end, first, marker genes were identified that were differentially expressed between splenic and peritoneal B-1a cells. By transfer experiments the question of whether this differential expression is due to the microenvironment or intrinsic to the particular cell population should be solved. Along this line novel markers for B-1 cells were intended to be defined. Secondly, a dominant antibody specificity that was repeatedly found amongst B-1 cells in a transgenic mouse was taken as specific marker for B-1 cells in order to better understand selective forces that act on such B-1 cells.

Analysis of gene expression in B-1 cells

Differentially expressed markers of B-1 cells: Several differences have been reported between peritoneal and splenic B-1 cells already (Rothstein, 2002). These results indicate that splenic and peritoneal B-1 cells differ phenotypically, biochemically and functionally. Two explanations can account for these differences: Either B-1 cells that reside in the spleen display an altered phenotype and function that is imprinted by the microenvironment of the spleen or splenic B-1 cells are of different developmental origin compared to peritoneal B-1 cells thus representing two alternative cell lineages. Genes expressed in peritoneal and splenic B-1 cells would therefore represent diagnostic patterns of independent developmental programs. In the first case, the gene expression pattern would depend on the location of the cells independent of their origin while in the second expression patterns would not be influenced by the microenvironment. Clearly, the first situation is true for genes analyzed in this work.

To achieve this conclusion, genes were identified on the microarrays that are strongly expressed in splenic B-1a cells but showed no or extremely low expression in peritoneal B-1a cells. Confirmation of the expression data obtained by arrays however yielded conflicting results in RT-PCR analysis. While Vcam1, Adamdec1 and Spic could be shown to fulfill the original criteria, data for Hpgd in peritoneal cells were contradictory. In three experiments using L2 or non-transgenic mice no Hpgd expression was observed while in another independent experiment with L2 mice expression was observed. Similarly, expression could be observed in peritoneal B-1

cells after cell transfer independent of the route of transfer. Hpgd encodes the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase an enzyme that is catalyzing the inactivation of prostaglandins and known to be downregulated during inflammation (Hahn et al., 1998; Ivanov et al., 2003). Therefore, expression of this gene might represent the inflammation status of the mouse rather than the influence of the microenvironment. The expression pattern of this gene was therefore excluded from the conclusions.

The expression pattern of two other genes (Mrc2, Lpl) also showed discrepancies between earlier and later RT-PCR experiments. Mrc2 encodes the mannose receptor (MR, CD206) which is involved in the clearance of endogenous glycoproteins as well as pathogen recognition (Taylor et al., 2005). Lpl encodes lipoprotein lipase (LPL) which promotes the hydrolysis of triacylglycerol and is involved in monocyte adhesion, induction of the gene for the inflammatory cytokine TNF-α and the promotion of cell proliferation (Mead et al., 2002).

Consistently, in splenic B-1a cells Mrc2 and Lpl expression was detectable in all experiments. In contrast, the initial RT-PCR of Mrc2 and Lpl indicated no or low expression in the peritoneal B-1a cells while in later experiments Mrc2 and Lpl were reproducibly detectable in such cells. The reason for this discrepancy is unclear. Possibly, the B-1a cells from peritoneum were contaminated by B-1b cells in which expression of these two genes could be detected on microarrays. On the other hand, a similar explanation as discussed for Hgpd might hold true also for Mrc2 and Lpl, expression simply reflecting the inflammatory status of the mice. Independent of the reason these two genes were excluded from the analysis.

Analysis of transferred B-1 cells: Cells from the peritoneal cavity of L2 mice were transferred into lymphocyte deficient Rag $1^{-/-}$ mice either by the i.v. or i.p. route. Unsorted cells were used since on one hand only B-1 cells are found in the peritoneum of such mice and on the other hand they contained T cells which might be essential for the migration of such B cells. $Rag1^{-/-}$ mice were used because no migration of B-1 cells to the spleen could be observed when normal or L2 mice were used as recipients for i.p. transfers (Kretschmer et al., 2003b). The lack of lymphocytes in the spleen of $Rag1^{-/-}$ mice obviously allows i.p. transferred B-1 cells to home to the spleen.

Independent of the route of transfer peritoneum derived B-1 cells could be found in the peritoneum as well as the spleen of recipient mice. In the case of i.p. transfer the course of colonization was followed. In both locations the number of B cells increased until three weeks after transfer and then remained roughly constant. A similar increase is found for T cells. This indicates that the transferred lymphocytes expand due to homeostatic proliferation (Woodland and Schmidt, 2005) until the compartments are filled at three weeks after transfer. The intrinsic feedback regulation then stops expansion and leaves the cells at a steady state number in both compartments.

Transferred cells were isolated and semiquantitative RT-PCR analysis performed for the expression of the selected diagnostic genes. Although only three appropriate genes could be included in this analysis (Vcam1, Adamdec1, Spic) unambiguous results were obtained. Low to no expression was observed in transferred B-1 cells in the peritoneum in accordance with expression before transfer. Such genes were found to be upregulated when the B-1 cells resided in the spleen after transfer. This suggests that the differential gene expression pattern observed for B-1 cells from spleen and peritoneum is largely influenced by the microenvironment. This finding however does not yet exclude the possibility that splenic and peritoneal B-1 are derived from two independent lineages but only indicates that peritoneal B-1 cells have the plasticity to express particular genes when migrating to the spleen of lymphopenic mice.

Clearly, this analysis needs to be extended to additional diagnostic genes for validation. In the present study only genes with known products were included while ESTs (expressed sequence tags) were ignored. Therefore, a follow up study should be extended to such gene products. In addition it would be important to analyze gene expression patterns of B-1 cells from spleen and peritoneum after transfer of splenic B-1 cells. This should give further hints on the plasticity of B-1 cells.

VCAM-1 expression on peritoneal B-1 cells: Particularly interesting results were obtained when the expression pattern of VCAM-1 was analyzed during the above experiments. Surprisingly, although no RNA expression of VCAM-1 was detectable in peritoneal B-1a cells on average 5% could be stained with an anti-VCAM-1 antibody. This staining has to be considered real since blockage of Fc-receptors by various means did not abolish or significantly lower the staining. Different hypotheses could

explain this finding: First, these cells could be migratory cells that have acquired VCAM-1 expression outside the peritoneal cavity and downregulated the gene expression upon entry into the peritoneum. Surface expression of the protein however would remain detectable for some time. Alternatively, shed VCAM-1 might have been absorbed passively by a fraction of B-1 cells. Shed VCAM-1 from stromal and stimulated endothelial cells has been observed (Kinashi et al., 1995) and is also found in serum under a broad range of disease conditions like atherosclerosis, rheumatoid arthritis or systemic lupus erythematosus (Gearing and Newman, 1993; Wellicome et al., 1993; Blankenberg et al., 2003). Nevertheless, even then one would expect that the VCAM-1 carrying peritoneal B-1 cells are migratory and recent immigrants into the peritoneum since otherwise all peritoneal B-1 cells should bear VCAM-1 on their surface.

To further investigate this phenomenon additional transfer experiments are indicated. So far only VCAM-1 negative B-1 cells from the peritoneum were transferred i.p and tested two weeks after. In further experiments earlier time point should be tested since at two weeks already steady state with regard to colonization and VCAM-1 expression might be reached. In addition, VCAM-1 positive cells should be transferred and followed in a similar way. If VCAM-1 is involved in the migration and homing of B-1 cells one would expect to find effects already early after transfer. Under these condition one would also expect that independent of the route of transfer and the phenotype of the transferred cells the ratio originally observed for VCAM-1^+/VCAM-1^- should be obtained when steady state is reached. These experiments should further support the idea that the microenvironment is defining the gene expression pattern of B-1 cells.

Effect of conditional Vcam1 deficiency on the B cell compartment: VCAM-1 is known to be constitutively expressed on bone marrow stromal/endothelial cells and classes of hematopoietic cells, like dendritic cells and macrophages (Freedman et al., 1990; Miyake et al., 1991; Koopman et al., 1991; Jacobsen et al., 1996; Schweitzer et al., 1996). Xia et al. furthermore showed constitutive VCAM-1 expression on human B lymphocytes from peripheral blood and plasmacytomas (Xia et al., 2001). The existence of VCAM-1 on B-1a cells described here is novel and its functional role on B cells is not known. VCAM-1 is thought to be involved in lymphocyte homing

(Papayannopoulou and Craddock, 1997) and is supposed to be a costimulatory molecule for T cells (Burkly et al., 1991).

To address which function VCAM-1 might have on B-1 cells, mice with a supposedly B cell specific Vcam1 deletion were investigated. Therefore, mice with floxed Vcam1 alleles were mated with CD19cre recombinant mice that express the Cre protein under the control of the CD19 promoter B cell specifically. No differences within the B cell compartment of such mice could be observed in comparison to control mice. However, flow cytometrical analysis of such mice revealed that VCAM-1 was still expressed on B cells. This is obviously due to a low efficiency of the CD19 driven Cre that has been observed also by other investigators (Rickert et al., 1997). In agreement, Southern blots of B cells from VCAM-$1^{flox/flox}$/CD19cre mice revealed bands of undeleted VCAM-1 genes. This suggests an incomplete deletion. Interestingly, in both VCAM-$1^{flox/flox}$/CD19cre and control mice on average 15% of the IgM^+ cells in the spleen were positive for VCAM-1 expression. This suggests for unknown reasons a strong selection of B cells with undeleted VCAM-1 into the VCAM-1 positive B cell fraction. In future, such incomplete deletions might be possible to be circumvented by employing a mouse line in which Cre is driven by the mb-1 promoter (the mb1 gene encodes the Igα protein). In such mice Cre efficiency is apparently 100% (Elias Hobeika, personal communication).

B-1 cell specific molecules: In flow cytometry B-1 cells are identified by a combination of surface markers that allows the distinction from other B cell subsets. For instance B-1 cells can be distinguished from follicular B-2 cells by their expression levels of IgM and IgD. B-1 cells display a $IgM^{hi}IgD^{lo}$ expression pattern whereas B-2 cells are IgM^+IgD^{hi}. The expression of the CD5 molecule in combination with IgM defines B-1a cells and distinguishes them from $CD5^-$ B-2 cells but also from B-1b cells. Other markers that characterize B-1 cells, in contrast to B-2 cells, are CD43 or Mac-1. However, the example of Mac-1 also shows the restrictions of such B-1 cell markers since Mac-1 defines well peritoneal B-1 cells but is not detected on splenic B-1 cells. Similarly, expression level of IgM and IgD can be used for the characterization of peritoneal cavity B-1 cells but is insufficient in the spleen because of the presence of other cell populations with an $IgM^{hi}IgD^{lo}$ phenotype like marginal zone B cells or fractions of immature B cells. Thus, in the spleen only CD5 represents a reliable

marker for B-1a cells. A unique pan B-1 cell marker would therefore be a valuable tool for studies of B-1 cells.

In this work it could be shown by RT-PCR that the F11r gene, encoding JAM-1, is expressed in B-1a cells in spleen and peritoneum of L2 mice. The expression of JAM-1 was confirmed by flow cytometrical analyses that were restricted to the lymphoid gate. Peritoneal B-1a cells of L2 mice were positive for JAM-1 staining. Similarly $IgM^{hi}CD5^{-}$ cells showed JAM-1 expression indicating that also B-1b cells express this marker. No expression could be detected on T cells or cells that were negative for IgM and CD5 staining. Thus, in the peritoneal cavity of L2 mice JAM-1 expression is only found on B-1 cells. Similarly, in non-transgenic mice, peritoneal cavity $IgM^{+}CD5^{+}$ B-1a cells were positive for JAM-1 and T cells and $IgM^{-}CD5^{-}$ cells were negative. B-1b and B-2 cells could be distinguished by the $IgM^{+}CD5^{lo}$ (B-2) and $IgM^{hi}CD5^{-}$ (B-1b) expression pattern and it could be shown, that JAM-1 was only expressed in the B-1b population.

In the spleen, T cells and $IgM^{-}CD5^{-}$ cells were negative for JAM-1 staining in both transgenic and control mice. Similarly, splenic B-2 cells in non-transgenic mice showed no JAM-1 expression. In contrast, B-1a cells in the spleen of L2 mice were clearly $JAM\text{-}1^{+}$. However, the staining was weaker compared to peritoneal cavity B-1a cells. The same was true for splenic B-1a cells of BALB/c mice.

Taken together, JAM-1 expression is detectable on all B-1 cells in transgenic and non-transgenic mice and not found on any other of the cell populations present in the lymphoid gate of spleen and peritoneal cavity. Thus, at these sites it can be considered a marker for B-1 cells in flow cytometry. It will be interesting to extend this analysis to other compartments like blood or gut associated tissue.

JAM-1 was originally characterized on platelets and was later found to be predominantly expressed in endothelial and epithelial intercellular junctions. The human orthologue of JAM-1 is expressed on a variety of circulating leukocytes in peripheral blood, including monocytes, neutrophils, B and T lymphocytes (Ebnet et al., 2004). On endothelia the interaction of JAM-1 with LFA-1 on leukocytes supports the adhesion and transendothelial migration (Ostermann et al., 2002). However, the role of JAM-1 expressed on leukocytes is not known and its function in B-1 cells has to be investigated.

A second gene, Adml, also qualified by RT-PCR as a potential B-1 cell marker. It was expressed in splenic and peritoneal B-1a cells of transgenic and non-transgenic L2

mice and showed only traces in B-2 cells. Adml encodes Adrenomedullin, which in the mouse is a small regulatory peptide 50 aa in length. It is expressed in a variety of cells and tissues e.g. heart, brain, kidney as well as in leukocytes in the blood like monocytes, mast cells but also in B and T cells. Initially it was characterized as a vasodilator but in the meantime many other functions have been reported, e.g. an impact on cell proliferation, hormone regulation and antimicrobial activity. A role as anti-apoptotic survival factor was also described (Ostermann et al., 2002). Preliminary experiments using a commercial anti-Adrenomedullin antibody did not reveal any intracellular staining of B-1 cells (data not shown). Therefore, it is not clear at the moment whether this molecule can be of use for B-1 cell studies. Clearly more systematic investigations need to be carried out in order to validate Adrenomedullin as a marker for B-1 cells especially in the light of a potential expression in non-B cells.

In summary, several novel markers have been identified so far which should in future either facilitate studies on B-1 cells or shed further light on the function of different subpopulations of B-1 cells.

The L2P3 antibody

The most important marker of a B cell and of a B-1 cell for that matter is its B cell receptor. According to the current hypothesis the BCR might imprint the B-1 phenotype onto a B cell (Lam and Rajewsky, 1999; Wang and Clarke, 2004b). Therefore, the study of the behavior of particular B-1 cell clones under various conditions should shed some light on the selective forces that act on this B cell. So far analysis of the BCRs of B-1 cells from L2 mice was restricted to sequence analysis. This has led to extremely interesting results already. A pronounced oligoclonality was observed among their BCR specificities with a few specificities dominating. Amongst those one was found in 15% of all peritoneal cavity B-1 cells and represented 10% of splenic B-1 cell specificities (Kretschmer et al., 2002; Kretschmer et al., 2003b). Two others represent 13% and 7,4% of the specificities in the peritoneal cavity. These specificities were B-1 cell specific as they could not be detected in B-2 cells so far. Thus they represent the appropriate invariant marker to further study such B-1 cells.

The 8H10 anti-idiotype antibody: By cell fusion using cells from spleen and peritoneal cavity of L2 mice a hybridoma could be established which secreted a monoclonal IgM - L2P3 - representing the specificity that was found at a 15% frequency in the peritoneum. This IgM was first used to establish a reagent - the anti-idiotypic antibody 8H10 – that to a large extend recognizes only antibodies with the dominating sequence. This was established by isolating B-1 cells that were recognized by the 8H10 antibody and analyzing their heavy chain sequence. 19 of such sequences were identical to L2P3. Four additional sequences displayed an identical CDR3 region but contained single aa exchanges. Such exchanges are characteristic for somatic hypermutation although B-1 cell derived antibodies usually lack somatic mutations (Berland and Wortis, 2002). Thus, such aa exchanges might be due to Taq-polymerase errors. Besides these sequences, four sequences were found that were deviating from the sequence of L2P3. Possibly the secondary antibody employed for sorting might be responsible for a contamination with B-1 cells not directly recognized by the anti-idiotypic antibody. Alternatively, although the sort was highly pure (98,4%) some residual non-L2P3 type of B-1 cells were present in the sample which might have contributed the non-L2P3 sequences. However, one also cannot exclude that the anti-idiotypic antibody recognizes these BCRs specifically via an idiotope that is shared but is not apparent from sequence comparison. A second sort and analysis should clarify this question. Independent of this, the $8H10^{+}$ antibody can be considered a highly specific anti-idiotypic antibody for the detection of the L2P3 specificity and should represent an extremely useful reagent.

The use of the 8H10 antibody in flow cytometrical analysis of the L2 mice revealed very interesting results. An obvious difference was observed when splenic and peritoneal B-1 cells from male and female mice were compared. Female mice contained significantly more B-1 cell of this specificity when directly compared with male mice. A discrepancy between the frequencies described originally for female L2 mice concerning this dominating specificity (Kretschmer et al., 2003b) and the present data was observed. This could be due to alterations in the hygienic conditions. However, since the size of the samples in the present work is still very small and pools of mice were used in the original work no importance was given to this discrepancy for the moment.

Differences in immune reactions between males and females are well established. Women are more prone to develop autoimmune diseases than men (Verthelyi, 2001; Bouman et al., 2005). Strong differences are also observed in the resistance of males and females mice towards *Listeria monocytogenes* infection (Lengeling personal communication). Therefore, the selective forces acting on B-1 cells from males and females might be different due to differences in resistance against the natural microbial environment. In addition, estrogen receptors are expressed in B cells (Grimaldi et al., 2005). Therefore, even a direct influence of the hormonal milieu on the selection of B-1 specificities can be envisioned.

Additional influences on the selection of the L2P3 specificity were observed when female mice from different housing conditions – SPF versus conventional - were compared. Lower numbers of B-1 cells stained with 8H10 were found in SPF mice that contain a defined commensal microbial flora in their intestine and are free of known bacterial, viral, and parasitic pathogens. The most obvious explanation for this observation is that microbes of the gut contribute significantly to the selection of B-1 specificities in the lymphoid compartments even when they are not associated with this organ.

Further experiments should now carefully extend the data on the impact of gender and housing. Defined pathogens with an intestinal port of entry should be tested in this context for their impact on the repertoire of B-1 derived antibodies in the various deep lymphoid compartments.

An influence of the age of the mice on the selection of the L2P3 specificity should also be tested since it is most likely not only driven by microbial antigens but also by autoantigen. For instance, Carmack et al. have reported a time-dependent accumulation of B-1 cells specific for a determinant on senescent erythrocytes (anti-BrMRBC, anti-PtC) (Carmack et al., 1990). Similarly, Hayakawa et al. observed a time-dependent increase of B-1 cells specific for the Thy-1 self-antigen (Hayakawa et al., 1999). The finding that L2P3 apparently binds to lymphoid cells in the peritoneum of L2 mice is in accordance with this. Although only a weak signal was observed the staining appeared to be specific as neither the second B-1 cell derived antibody L2M15 nor MOPC104E reacted with these cells and no binding of L2P3 was observed in other organs. This might indicate the existence of an self-antigenic determinant recognized by L2P3. So far the cells bound by L2P3 have not been

characterized. As most (~80%) of the cells in the lymphoid gate of the peritoneal cavity are B cells, they mainly contribute to the observed staining.

Unexplained is still why this antigen is only detected in L2 mice but different possibilities exist concerning its nature. Either it is expressed endogenously i.e. by the cells themselves like in the case of the autoantigen Thy-1 (Hayakawa et al., 1999) which is expressed on T cells. Alternatively, it could be a soluble antigen attached to receptors on the surface of these cells. An example that even a BCR can be involved was provided by Carmack et al. (Carmack et al., 1990). When a B-1 cell derived antibody (anti-BrMRBC) was used to stain peritoneal cavity cells, a fraction of B-1 cells bound the antibody. More then half of the BCRs of these B-1 cells were specific for BrMRBC. This suggests that the self-antigen was bound by the BCR of these particular B-1 cells and was then detected by the antibody used in the analysis in a sandwich kind of staining. Thus, one could envision that L2P3 detects a determinant on an antibody that is bound to an self antigen on peritoneal cells of L2 mice. However, more likely is that L2P3 is specific for an intracellular antigen as it is known for other natural antibodies (Baumgarth et al., 2005). The physiology of the L2 mice, for instance apoptosis or necrosis of particular cells, might solubilize this antigen and render it detectable on the peritoneal lymphocytes of such mice. This needs to be further worked out in a systematic way. Intracellular staining will have to be carried out using the L2P3 antibody. As samples, cytological and histological preparations of cells and tissues from L2 and control mice should be used. Apoptotic cells should be included in this analysis.

Consistent with the detection of a self antigen was the in vivo behavior of the IgM antibody L2P3 when injected into L2 mice and compared with two other B-1 cell derived IgM – L2M15 and MOPC104E as well as one B-2 cell derived antibody 2D4. The 2D4 antibody showed a half-live of 22 h similar to the half-life of IgM described by others (Vieira and Rajewsky, 1988). In contrast L2P3, L2M15 and MOPC104E showed a pronounced bi-phasic behavior in their clearance rates. During the first phase of clearance the half-live of these antibodies was around 10 h which is short compared to 2D4. In the second phase the half-life was increased to 35-43 h. The monophasic kinetic of 2D4 is compatible with the expected short half-live of an IgM antibody which is rather short compared to IgG antibodies (Vieira and Rajewsky, 1988) and probably reflects the natural degradation rate of this class of antibodies. In contrast the B-1 cell derived antibodies are most likely submitted to two processes.

The first process indicated by a short-half life might reflect the binding of these antibodies to self antigen leading to their rapid clearance from sera. This binding might result in the clearance or sequestration of the antigen thus denying further access to the anybodies. With the loss of the antigen the antibody will be cleared at its natural rate which is reflected by a extended half-live in serum.
It is interesting that all B-1 cell derived antibodies in this experiments behaved similarly indicating that they all might bind to self-antigenic determinants although only for L2P3 a weak self-reactivity could be demonstrated in flow cytometrical analysis. Analyses of these antibodies so far only tested a fraction of the putative autoantigen presenting cells, as only cells from spleen, thymus, bone marrow and peritoneum were analyzed. Therefore, self-reactivity as an explanation for the biphasic clearance rates of the B-1 derived antibodies is consistent with the data. However, there is one technical draw back at the moment. Only few time points were taken for analysis thus a similar curve for 2D4 might have been missed due to the limited data available for calculation. Thus, clearly these experiments need to be repeated with a more detailed analysis at the early phase of the clearance. In addition, non transgenic mice should be included in this analysis. Furthermore, histological analysis of various tissues shortly after administration of the antibody should be carried out since it should be possible to find cells with bound antibody if a self-reactivity exists. This might also help to identify the self-antigen itself.

Taken together, several novel aspects of the physiology of B-1 cells were addressed during this work. It became clear during the course of the experiments that this B cell population is dynamically reacting to environmental changes. This was most clearly demonstrated by the difference in the frequency of L2P3 idiotype bearing B-1 cells in mice from different housing conditions. Therefore, some of the analyses were aggravated by such changes that could not be controlled but on the other hand might reflect the natural situation that should be further analyzed. Nevertheless, interesting information on the difference between splenic and peritoneal B-1 cell as well as on the selective forces acting on them could be obtained which shed further light on this still enigmatic B cell population.

5 Summary

In the present work the transgenic mouse L2 was used in which almost exclusively B-1 cells are found in the spleen and peritoneum. Several features of B-1 cells were investigated in such mice. First, the question was addressed if differential gene expression observed between splenic and peritoneal derived B-1a cells is due to the distinct localization or due to different developmental origin of these cells. Based on microarray data genes were established by RT-PCR analyses, that were expressed in splenic B-1a cells and low or absent in peritoneal B-1a cells. These genes were used as diagnostic markers in transfer experiments. In these experiments peritoneal derived B-1a cells were injected into lymphopenic mice and reisolated from the spleen as well as the peritoneal cavity. Then the gene expression pattern of such cells was analyzed. All of the diagnostic marker genes were upregulated in peritoneal derived B-1 cells that resided in the spleen. Thus it could be demonstrated that the microenvironment is imprinting the gene expression pattern in such B-1 cells.

In line with the gene expression analysis of B-1 cells JAM-1 was identified as a novel marker for B-1 cells located in spleen and peritoneum of mice. This protein was not expressed in B-2 cells, T cells or any other population at these sites and therefore specifically defines B-1 cells in flow cytometrical analysis in spleen and peritoneum.

In the last part of this work an anti-idiotypic antibody directed against a dominant B-1 cell specificity that had been identified on B-1 cells of L2 mice before, was generated. To achieve this, hybridomas of splenic and peritoneal B-1a cells from L2 mice were established that produced monoclonal IgM antibodies. Sequence analysis of cDNA of V_H regions from the individual clones was carried out. One antibody, L2P3, was of particular interest because it represented one of the dominant specificities in the B-1 cell pool of L2 mice. A second antibody, L2M15, represented a specificity only occasionally found in blood derived B-1 cells of L2 mice before and was used as control antibody for the experiments. L2P3 was used to generate an anti-idiotypic antibody in rats which was termed 8H10. Stainings performed with this anti-idiotype indicate that the generation and maintenance of L2P3 positive cells might be subjected to different selectional forces. Higher numbers of $L2P3^+$ cells were detected in female mice compared to male mice. Furthermore, the microbial environment might have an impact on the selection as fewer $L2P3^+$ cells were

observed in SPF mice than in mice housed under conventional conditions. The anti-idiotype generated during this work is therefore a valuable tool for the investigation of selectional forces B-1 cells are submitted to. Further work with this antibody will help to unravel the impact of individual selectional influences shaping the B-1 cell pool in L2 mice.

Finally, the purified antibodies L2P3 and L2M15 were further characterized. The binding of L2P3 on cells in the lymphoid gate of the peritoneal cavity revealed binding to an autoantigenic determinant whereas such binding was not observed for L2M15. An analysis of antibody clearance rates from sera of L2 mice extended the characterization of these antibodies. Biphasic clearance rates were found for the B-1 cell derived antibodies which is consistent with the idea that they bind to an autoantigen in L2 mice.

6 References

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E. (2001). Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659-669.

Ahearn, J.M., Fischer, M.B., Croix, D., Goerg, S., Ma, M., Xia, J., Zhou, X., Howard, R.G., Rothstein, T.L., and Carroll, M.C. (1996). Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity. *4*, 251-262.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Allman, D., Srivastava, B., and Lindsley, R.C. (2004). Alternative routes to maturity: branch points and pathways for generating follicular and marginal zone B cells. Immunol. Rev. *197*, 147-160.

Araki, M., Araki, K., and Vassalli, P. (1993). Cloning and sequencing of mouse VCAM-1 cDNA 5. Gene *126*, 261-264.

Arnold, L.W., Pennell, C.A., McCray, S.K., and Clarke, S.H. (1994). Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. J. Exp. Med. *179*, 1585-1595.

Arp, B., McMullen, M.D., and Storb, U. (1982). Sequences of immunoglobulin lambda 1 genes in a lambda 1 defective mouse strain. Nature *298*, 184-187.

Barreto, V. and Cumano, A. (2000). Frequency and characterization of phenotypic Ig heavy chain allelically included IgM-expressing B cells in mice. J. Immunol. *164*, 893-899.

Bassing, C.H., Swat, W., and Alt, F.W. (2002). The mechanism and regulation of chromosomal V(D)J recombination. Cell *109 Suppl*, S45-S55.

Baumgarth, N., Chen, J., Herman, O.C., Jager, G.C., and Herzenberg, L.A. (2000). The role of B-1 and B-2 cells in immune protection from influenza virus infection. Curr. Top. Microbiol. Immunol *252*, 163-169.

Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L., Herzenberg, L.A., and Herzenberg, L.A. (1999). Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc. Natl. Acad. Sci. U. S A *96*, 2250-2255.

Baumgarth, N., Tung, J.W., and Herzenberg, L.A. (2005). Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Semin. Immunopathol.

Benedict, C.L., Gilfillan, S., Thai, T.H., and Kearney, J.F. (2000). Terminal deoxynucleotidyl transferase and repertoire development. Immunol Rev *175*, 150-157.

Bentolila, L.A., Olson, S., Marshall, A., Rougeon, F., Paige, C.J., Doyen, N., and Wu, G.E. (1999). Extensive junctional diversity in Ig light chain genes from early B cell progenitors of mu MT mice 1. J. Immunol *162*, 2123-2128.

Berland, R. and Wortis, H.H. (2002). Origins and functions of B-1 cells with notes on the role of CD5. Annu. Rev. Immunol. *20*, 253-300.

Besmer, E., Gourzi, P., and Papavasiliou, F.N. (2004). The regulation of somatic hypermutation. Curr. Opin. Immunol *16*, 241-245.

Bikah, G., Carey, J., Ciallella, J.R., Tarakhovsky, A., and Bondada, S. (1996). CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. Science *274*, 1906-1909.

Blankenberg, S., Barbaux, S., and Tiret, L. (2003). Adhesion molecules and atherosclerosis. Atherosclerosis *170*, 191-203.

Blomberg, B. and Tonegawa, S. (1982). DNA sequences of the joining regions of mouse lambda light chain immunoglobulin genes. Proc. Natl. Acad. Sci. U. S A *79*, 530-533.

Bos, N.A., Meeuwsen, C.G., Wostmann, B.S., Pleasants, J.R., and Benner, R. (1988). The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. Cell Immunol *112*, 371-380.

Bouman, A., Heineman, M.J., and Faas, M.M. (2005). Sex hormones and the immune response in humans. Hum. Reprod. Update.

Bradl, H. and Jack, H.M. (2001). Surrogate light chain-mediated interaction of a soluble pre-B cell receptor with adherent cell lines. J. Immunol *167*, 6403-6411.

Briles, D.E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J., and Barletta, R. (1981). Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. J. Exp. Med. *153*, 694-705.

Bryant, P. and Ploegh, H. (2004). Class II MHC peptide loading by the professionals. Curr. Opin. Immunol *16*, 96-102.

Burkly, L.C., Jakubowski, A., Newman, B.M., Rosa, M.D., Chi-Rosso, G., and Lobb, R.R. (1991). Signaling by vascular cell adhesion molecule-1 (VCAM-1) through VLA-4 promotes CD3-dependent T cell proliferation. Eur. J. Immunol *21*, 2871-2875.

Busslinger, M. (2004). Transcriptional control of early B cell development. Annu. Rev Immunol *22*, 55-79.

Calame, K.L., Lin, K.I., and Tunyaplin, C. (2003). Regulatory mechanisms that determine the development and function of plasma cells. Annu. Rev. Immunol. *21*, 205-230.

Carmack, C.E., Shinton, S.A., Hayakawa, K., and Hardy, R.R. (1990). Rearrangement and selection of VH11 in the Ly-1 B cell lineage
1. J. Exp. Med. *172*, 371-374.

Carsetti, R., Rosado, M.M., and Wardmann, H. (2004). Peripheral development of B cells in mouse and man. Immunol. Rev. *197*, 179-191.

Casadevall, A. and Pirofski, L.A. (2004). New concepts in antibody-mediated immunity. Infect. Immun. *72*, 6191-6196.

Casali, P., Burastero, S.E., Nakamura, M., Inghirami, G., and Notkins, A.L. (1987). Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. Science *236*, 77-81.

Chan, V.W., Meng, F., Soriano, P., DeFranco, A.L., and Lowell, C.A. (1997). Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity. *7*, 69-81.

Chaudhuri, J. and Alt, F.W. (2004). Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. Nat. Rev Immunol *4*, 541-552.

Chevillard, C., Ozaki, J., Herring, C.D., and Riblet, R. (2002). A three-megabase yeast artificial chromosome contig spanning the C57BL mouse Igh locus. J. Immunol. *168*, 5659-5666.

Chowdhury, D. and Sen, R. (2004). Regulation of immunoglobulin heavy-chain gene rearrangements. Immunol. Rev. *200*, 182-196.

Christensen, J.L. and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc. Natl. Acad. Sci. U. S A *98*, 14541-14546.

Chumley, M.J., Dal Porto, J.M., Kawaguchi, S., Cambier, J.C., Nemazee, D., and Hardy, R.R. (2000). A VH11V kappa 9 B cell antigen receptor drives generation of CD5+ B cells both in vivo and in vitro. J. Immunol. *164*, 4586-4593.

Claflin, J.L. and Berry, J. (1988). Genetics of the phosphocholine-specific antibody response to Streptococcus pneumoniae. Germ-line but not mutated T15 antibodies are dominantly selected. J. Immunol *141*, 4012-4019.

Claflin, J.L., Lieberman, R., and Davie, J.M. (1974). Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. J. Exp. Med. *139*, 58-73.

Clarke, S.H. and Arnold, L.W. (1998). B-1 cell development: evidence for an uncommitted immunoglobulin (Ig)M+ B cell precursor in B-1 cell differentiation. J. Exp. Med. *187*, 1325-1334.

Cong, Y.Z., Rabin, E., and Wortis, H.H. (1991). Treatment of murine CD5- B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. Int. Immunol. *3*, 467-476.

Cook-Mills, J.M. (2002). VCAM-1 signals during lymphocyte migration: role of reactive oxygen species. Mol. Immunol *39*, 499-508.

Cornall, R.J., Goodnow, C.C., and Cyster, J.G. (1995). The regulation of self-reactive B cells. Curr. Opin. Immunol. *7*, 804-811.

Cumano, A., Paige, C.J., Iscove, N.N., and Brady, G. (1992). Bipotential precursors of B cells and macrophages in murine fetal liver. Nature *356*, 612-615.

Damle, N.K., Klussman, K., Linsley, P.S., and Aruffo, A. (1992). Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. J. Immunol *148*, 1985-1992.

Dauphinee, M., Tovar, Z., and Talal, N. (1988). B cells expressing CD5 are increased in Sjogren's syndrome 1. Arthritis Rheum. *31*, 642-647.

De, P., Peak, M.M., and Rodgers, K.K. (2004). DNA cleavage activity of the V(D)J recombination protein RAG1 is autoregulated. Mol. Cell Biol. *24*, 6850-6860.

DeFranco, A.L. (1999). Distinct types of antibody responses. In *Fundamental immunology* (Edited by Paul W.E.), p. 241, Lippincott-Raven Publishers, Philadelphia.

Diaz, M. and Casali, P. (2002). Somatic immunoglobulin hypermutation. Curr. Opin. Immunol *14*, 235-240.

Doody, G.M., Bell, S.E., Vigorito, E., Clayton, E., McAdam, S., Tooze, R., Fernandez, C., Lee, I.J., and Turner, M. (2001). Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. Nat. Immunol. *2*, 542-547.

Douagi, I., Vieira, P., and Cumano, A. (2002). Lymphocyte commitment during embryonic development, in the mouse. Semin. Immunol. *14*, 361-369.

Düber, S. B cell development in fetal liver and adult bone marrow. 29-10-2004.
Ref Type: Generic

Duber, S., Engel, H., Rolink, A., Kretschmer, K., and Weiss, S. (2003). Germline transcripts of immunoglobulin light chain variable regions are structurally diverse and differentially expressed. Mol. Immunol *40*, 509-516.

Dul, J.L., Argon, Y., Winkler, T., ten Boekel, E., Melchers, F., and Martensson, I.L. (1996). The murine VpreB1 and VpreB2 genes both encode a protein of the surrogate light chain and are co-expressed during B cell development. Eur. J. Immunol *26*, 906-913.

Early, P., Huang, H., Davis, M., Calame, K., and Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. Cell *19*, 981-992.

Ebnet, K., Suzuki, A., Ohno, S., and Vestweber, D. (2004). Junctional adhesion molecules (JAMs): more molecules with dual functions?
1. J. Cell Sci. *117*, 19-29.

Edry, E. and Melamed, D. (2004). Receptor editing in positive and negative selection of B lymphopoiesis. J. Immunol *173*, 4265-4271.

Engel, H., Bogen, B., Muller, U., Andersson, J., Rolink, A., and Weiss, S. (1998). Expression level of a transgenic lambda2 chain results in isotype exclusion and commitment to B1 cells. Eur. J. Immunol. *28*, 2289-2299.

Engel, H., Rolink, A., and Weiss, S. (1999). B cells are programmed to activate kappa and lambda for rearrangement at consecutive developmental stages. Eur. J. Immunol. *29*, 2167-2176.

Engel, P., Zhou, L.J., Ord, D.C., Sato, S., Koller, B., and Tedder, T.F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. *3*, 39-50.

Feeney, A.J. (1990). Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J. Exp. Med. *172*, 1377-1390.

Fischer, G.M., Solt, L.A., Hastings, W.D., Yang, K., Gerstein, R.M., Nikolajczyk, B.S., Clarke, S.H., and Rothstein, T.L. (2001). Splenic and peritoneal B-1 cells differ in terms of transcriptional and proliferative features that separate peritoneal B-1 from splenic B-2 cells. Cell Immunol. *213*, 62-71.

Forster, I., Gu, H., and Rajewsky, K. (1988). Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. EMBO J. *7*, 3693-3703.

Forster, I. and Rajewsky, K. (1987). Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. Eur. J. Immunol *17*, 521-528.

Franklin, A. and Blanden, R.V. (2004). On the molecular mechanism of somatic hypermutation of rearranged immunoglobulin genes
1. Immunol Cell Biol. *82*, 557-567.

Frazer, J.K. and Capra, J.D. (1999). Immunoglobulins: Structure and Function. In *Fundamental immunology* (Edited by Paul W.E.), pp. 37-74, Lippincott-Raven, Philadelphia.

Freedman, A.S., Munro, J.M., Rice, G.E., Bevilacqua, M.P., Morimoto, C., McIntyre, B.W., Rhynhart, K., Pober, J.S., and Nadler, L.M. (1990). Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110
2. Science *249*, 1030-1033.

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W., and Cantley, L.C. (1999). Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. Science *283*, 393-397.

Gauld, S.B., Dal Porto, J.M., and Cambier, J.C. (2002). B cell antigen receptor signaling: roles in cell development and disease. Science *296*, 1641-1642.

Gauthier, L., Rossi, B., Roux, F., Termine, E., and Schiff, C. (2002). Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. Proc. Natl. Acad. Sci. U. S A *99*, 13014-13019.

Gearing, A.J. and Newman, W. (1993). Circulating adhesion molecules in disease. Immunol Today *14*, 506-512.

Gerdes, T. and Wabl, M. (2002). Physical map of the mouse lambda light chain and related loci. Immunogenetics *54*, 62-65.

Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C., and Mathis, D. (1993). Mice lacking TdT: mature animals with an immature lymphocyte repertoire. Science *261*, 1175-1178.

Godin, I.E., Garcia-Porrero, J.A., Coutinho, A., Dieterlen-Lievre, F., and Marcos, M.A. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature *364*, 67-70.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., and . (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676-682.

Grawunder, U., Leu, T.M., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F., and Winkler, T.H. (1995a). Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity. *3*, 601-608.

Grawunder, U., Rolink, A., and Melchers, F. (1995b). Induction of sterile transcription from the kappa L chain gene locus in V(D)J recombinase-deficient progenitor B cells. Int. Immunol *7*, 1915-1925.

Greenspan, N.S. and Bona, C.A. (1993). Idiotypes: structure and immunogenicity. FASEB J. *7*, 437-444.

Grimaldi, C.M., Hicks, R., and Diamond, B. (2005). B cell selection and susceptibility to autoimmunity 1. J. Immunol *174*, 1775-1781.

Hahn, E.L., Clancy, K.D., Tai, H.H., Ricken, J.D., He, L.K., and Gamelli, R.L. (1998). Prostaglandin E2 alterations during sepsis are partially mediated by endotoxin-induced inhibition of prostaglandin 15-hydroxydehydrogenase. J. Trauma *44*, 777-781.

Hamilton, A.M. and Kearney, J.F. (1994). Effects of IgM allotype suppression on serum IgM levels, B-1 and B-2 cells, and antibody responses in allotype heterozygous F1 mice. Dev. Immunol. *4*, 27-41.

Hardy, R.R., Carmack, C.E., Shinton, S.A., Kemp, J.D., and Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med. *173*, 1213-1225.

Hardy, R.R. and Hayakawa, K. (2004). Development of B cells producing natural autoantibodies to thymocytes and senescent erythrocytes. Springer Semin. Immunopathol.

Hardy, R.R., Wasserman, R., Li, Y.S., Shinton, S.A., and Hayakawa, K. (2000). Response by B cell precursors to pre-B receptor assembly: differences between fetal liver and bone marrow. Curr. Top. Microbiol. Immunol *252*, 25-30.

Hardy, R.R., Wei, C.J., and Hayakawa, K. (2004). Selection during development of VH11+ B cells: a model for natural autoantibody-producing CD5+ B cells. Immunol Rev *197*, 60-74.

Hartley, S.B., Cooke, M.P., Fulcher, D.A., Harris, A.W., Cory, S., Basten, A., and Goodnow, C.C. (1993). Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell *72*, 325-335.

Hashimoto, A., Takeda, K., Inaba, M., Sekimata, M., Kaisho, T., Ikehara, S., Homma, Y., Akira, S., and Kurosaki, T. (2000). Cutting edge: essential role of phospholipase C-gamma 2 in B cell development and function. J. Immunol. *165*, 1738-1742.

Haughton, G., Arnold, L.W., Whitmore, A.C., and Clarke, S.H. (1993). B-1 cells are made, not born. Immunol Today *14*, 84-87.

Haury, M., Sundblad, A., Grandien, A., Barreau, C., Coutinho, A., and Nobrega, A. (1997). The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. Eur. J. Immunol *27*, 1557-1563.

Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Allman, D., Stewart, C.L., Silver, J., and Hardy, R.R. (1999). Positive selection of natural autoreactive B cells. Science *285*, 113-116.

Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Wen, L.J., Dashoff, J., and Hardy, R.R. (2003). Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. J. Exp. Med. *197*, 87-99.

Hayakawa, K., Hardy, R.R., Honda, M., Herzenberg, L.A., Steinberg, A.D., and Herzenberg, L.A. (1984). Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proc. Natl. Acad. Sci. U. S A *81*, 2494-2498.

Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A. (1983). The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice
2. J. Exp. Med. *157*, 202-218.

Hayakawa, K., Tarlinton, D., and Hardy, R.R. (1994). Absence of MHC class II expression distinguishes fetal from adult B lymphopoiesis in mice. J. Immunol. *152*, 4801-4807.

Hendriks, R.W., de Bruijn, M.F., Maas, A., Dingjan, G.M., Karis, A., and Grosveld, F. (1996). Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. EMBO J. *15*, 4862-4872.

Hendriks, R.W. and Middendorp, S. (2004). The pre-BCR checkpoint as a cell-autonomous proliferation switch. Trends Immunol *25*, 249-256.

Herzenberg, L.A. (2000). B-1 cells: the lineage question revisited. Immunol. Rev. *175*, 9-22.

Herzenberg, L.A., Stall, A.M., Lalor, P.A., Sidman, C., Moore, W.A., Parks, D.R., and Herzenberg, L.A. (1986). The Ly-1 B cell lineage. Immunol Rev *93*, 81-102.

Hiramatsu, R., Akagi, K., Matsuoka, M., Sakumi, K., Nakamura, H., Kingsbury, L., David, C., Hardy, R.R., Yamamura, K., and Sakano, H. (1995). The 3' enhancer region determines the B/T specificity and pro-B/pre-B specificity of immunoglobulin V kappa-J kappa joining
1. Cell *83*, 1113-1123.

Hofmann, M., Nussbaum, A.K., Emmerich, N.P., Stoltze, L., and Schild, H. (2001). Mechanisms of MHC class I-restricted antigen presentation. Expert. Opin. Ther. Targets. *5*, 379-393.

Howie, J.B. and Helyer, B.J. (1968). The immunology and pathology of NZB mice
1. Adv. Immunol *9*, 215-266.

Humbert, P.O. and Corcoran, L.M. (1997). oct-2 gene disruption eliminates the peritoneal B-1 lymphocyte lineage and attenuates B-2 cell maturation and function. J. Immunol. *159*, 5273-5284.

Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N., and Kincade, P.W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity *17*, 117-130.

Ishiyama, N., Kitagawa, M., Takahashi, H., Kina, T., and Hirokawa, K. (1998). Expression of VCAM-1 in lymphocytes during the process of apoptosis. Pathobiology *66*, 274-283.

Ivanov, A.I., Scheck, A.C., and Romanovsky, A.A. (2003). Expression of genes controlling transport and catabolism of prostaglandin E2 in lipopolysaccharide fever. Am. J. Physiol Regul. Integr. Comp Physiol *284*, R698-R706.

Jackson, S.P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis *23*, 687-696.

Jacobsen, K., Kravitz, J., Kincade, P.W., and Osmond, D.G. (1996). Adhesion receptors on bone marrow stromal cells: in vivo expression of vascular cell adhesion molecule-1 by reticular cells and sinusoidal endothelium in normal and gamma-irradiated mice
3. Blood *87*, 73-82.

Johansen, F.E., Braathen, R., and Brandtzaeg, P. (2000). Role of J chain in secretory immunoglobulin formation. Scand. J. Immunol *52*, 240-248.

Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M., and Nielsen, P.J. (1999). Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. Immunity. *11*, 547-554.

Jung, D. and Alt, F.W. (2004). Unraveling V(D)J recombination; insights into gene regulation. Cell *116*, 299-311.

Kabat E.A., Wu T.T., Reid-Miller M., Perry H.M. and Gottesman K.M. (1987) *Sequences of proteins of immunological interest*.

Kantor, A.B., Stall, A.M., Adams, S., Herzenberg, L.A., and Herzenberg, L.A. (1992). Differential development of progenitor activity for three B-cell lineages. Proc. Natl. Acad. Sci. U. S A *89*, 3320-3324.

Karasuyama, H., Rolink, A., Shinkai, Y., Young, F., Alt, F.W., and Melchers, F. (1994). The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. Cell *77*, 133-143.

Kawaguchi, S. (1988). Reactivity of mouse antibodies against bromelain-treated mouse erythrocytes with various mouse cells before and after protease treatment. Int. Arch. Allergy Appl. Immunol *86*, 458-461.

Kawaguchi, S. (1989). Reactivity of mouse antibodies against bromelain-treated mouse erythrocytes with thrombin-treated mouse platelets. Immunology *66*, 335-338.

Kawamoto, H., Ikawa, T., Ohmura, K., Fujimoto, S., and Katsura, Y. (2000). T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. Immunity. *12*, 441-450.

Kearney, J.F., Radbruch, A., Liesegang, B., and Rajewsky, K. (1979). A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines
2. J. Immunol *123*, 1548-1550.

Kearney, J.F., Vakil, M., and Dwyer, D.S. (1987). Idiotypes and autoimmunity. Ciba Found. Symp. *129*, 109-122.

Kenter, A.L. and Tredup, J. (1991). High expression of a 3'----5' exonuclease activity is specific to B lymphocytes. Mol. Cell Biol. *11*, 4398-4404.

Khan, W.N., Alt, F.W., Gerstein, R.M., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A.B., Herzenberg, L.A., and . (1995). Defective B cell development and function in Btk-deficient mice. Immunity. *3*, 283-299.

Kinashi, T., St Pierre, Y., and Springer, T.A. (1995). Expression of glycophosphatidylinositol-anchored and -non-anchored isoforms of vascular cell adhesion molecule 1 in murine stromal and endothelial cells. J. Leukoc. Biol. *57*, 168-173.

Kleinfield, R.W. and Weigert, M.G. (1989). Analysis of VH gene replacement events in a B cell lymphoma. J. Immunol *142*, 4475-4482.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Koopman, G., Keehnen, R.M., Lindhout, E., Newman, W., Shimizu, Y., van Seventer, G.A., de Groot, C., and Pals, S.T. (1994). Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J. Immunol *152*, 3760-3767.

Koopman, G., Keehnen, R.M., Lindhout, E., Zhou, D.F., de Groot, C., and Pals, S.T. (1997). Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. Eur. J. Immunol *27*, 1-7.

Koopman, G., Parmentier, H.K., Schuurman, H.J., Newman, W., Meijer, C.J., and Pals, S.T. (1991). Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways
1. J. Exp. Med. *173*, 1297-1304.

Kretschmer, K., Engel, H., and Weiss, S. (2002). Strong antigenic selection shaping the immunoglobulin heavy chain repertoire of B-1a lymphocytes in lambda 2(315) transgenic mice. Eur. J. Immunol *32*, 2317-2327.

Kretschmer, K., Jungebloud, A., Stopkowicz, J., Kleinke, T., Hoffmann, R., and Weiss, S. (2003a). The selection of marginal zone B cells differs from that of B-1a cells. J. Immunol *171*, 6495-6501.

Kretschmer, K., Jungebloud, A., Stopkowicz, J., Stoermann, B., Hoffmann, R., and Weiss, S. (2003b). Antibody repertoire and gene expression profile: implications for different developmental and functional traits of splenic and peritoneal B-1 lymphocytes. J. Immunol *171*, 1192-1201.

Kretschmer, K., Stopkowicz, J., Scheffer, S., Greten, T.F., and Weiss, S. (2004). Maintenance of peritoneal B-1a lymphocytes in the absence of the spleen. J. Immunol *173*, 197-204.

Kudo, A. and Melchers, F. (1987). A second gene, VpreB in the lambda 5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. EMBO J. *6*, 2267-2272.

Lacaud, G., Carlsson, L., and Keller, G. (1998). Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity. *9*, 827-838.

Lafaille, J.J., DeCloux, A., Bonneville, M., Takagaki, Y., and Tonegawa, S. (1989). Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining
1. Cell *59*, 859-870.

Lalor, P.A., Herzenberg, L.A., Adams, S., and Stall, A.M. (1989a). Feedback regulation of murine Ly-1 B cell development. Eur. J. Immunol. *19*, 507-513.

Lalor, P.A., Stall, A.M., Adams, S., and Herzenberg, L.A. (1989b). Permanent alteration of the murine Ly-1 B repertoire due to selective depletion of Ly-1 B cells in neonatal animals. Eur. J. Immunol. *19*, 501-506.

Lam, K.P., Kuhn, R., and Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell *90*, 1073-1083.

Lam, K.P. and Rajewsky, K. (1999). B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development
2. J. Exp. Med. *190*, 471-477.

Lam, K.P. and Stall, A.M. (1994). Major histocompatibility complex class II expression distinguishes two distinct B cell developmental pathways during ontogeny. J. Exp. Med. *180*, 507-516.

Landau, N.R., Schatz, D.G., Rosa, M., and Baltimore, D. (1987). Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol. Cell Biol. *7*, 3237-3243.

Lee, G.S., Brandt, V.L., and Roth, D.B. (2004). B cell development leads off with a base hit: dU:dG mismatches in class switching and hypermutation. Mol. Cell *16*, 505-508.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S., and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase cbeta-deficient mice. Science *273*, 788-791.

Li, Y.S., Hayakawa, K., and Hardy, R.R. (1993). The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. *178*, 951-960.

Li, Y.S., Wasserman, R., Hayakawa, K., and Hardy, R.R. (1996). Identification of the earliest B lineage stage in mouse bone marrow. Immunity *5*, 527-535.

Lindhout, E., Mevissen, M.L., Kwekkeboom, J., Tager, J.M., and de Groot, C. (1993). Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre B cells from death by apoptosis. Clin. Exp. Immunol *91*, 330-336.

Livak, F. (2004). In vitro and in vivo studies on the generation of the primary T-cell receptor repertoire. Immunol Rev *200*, 23-35.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C., and Carsetti, R. (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J. Exp. Med. *190*, 75-89.

Lopes-Carvalho, T. and Kearney, J.F. (2004). Development and selection of marginal zone B cells. Immunol. Rev. *197*, 192-205.

Martin, F. and Kearney, J.F. (2001). B1 cells: similarities and differences with other B cell subsets. Curr. Opin. Immunol. *13*, 195-201.

Martin, F., Oliver, A.M., and Kearney, J.F. (2001). Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity *14*, 617-629.

Martin, W.J. and Martin, S.E. (1975). Thymus reactive IgM autoantibodies in normal mouse sera. Nature *254*, 716-718.

Masmoudi, H., Mota-Santos, T., Huetz, F., Coutinho, A., and Cazenave, P.A. (1990). All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. Int. Immunol *2*, 515-520.

Max, E. E. Immunoglobulins: Molecular Genetics. Paul W.E. Fundamental Immunology, 111-182. 1999. Lippincott-Raven.
Ref Type: Generic

Mc Heyzer-Williams L.J. (2005). Antigen-specific memory B cell development. Annu. Rev. Immunol. *23*, 487.

McCormack, W.T., Tjoelker, L.W., Carlson, L.M., Petryniak, B., Barth, C.F., Humphries, E.H., and Thompson, C.B. (1989). Chicken IgL gene rearrangement involves deletion of a circular episome and addition of single nonrandom nucleotides to both coding segments
1. Cell *56*, 785-791.

McHeyzer-Williams, M.G. (2003). B cells as effectors. Curr. Opin. Immunol. *15*, 354-361.

Mead, J.R., Irvine, S.A., and Ramji, D.P. (2002). Lipoprotein lipase: structure, function, regulation, and role in disease. J. Mol. Med. *80*, 753-769.

Mebius, R.E., Miyamoto, T., Christensen, J., Domen, J., Cupedo, T., Weissman, I.L., and Akashi, K. (2001). The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. J. Immunol. *166*, 6593-6601.

Mercolino, T.J., Arnold, L.W., and Haughton, G. (1986). Phosphatidyl choline is recognized by a series of Ly-1+ murine B cell lymphomas specific for erythrocyte membranes. J. Exp. Med. *163*, 155-165.

Mercolino, T.J., Arnold, L.W., Hawkins, L.A., and Haughton, G. (1988). Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. J. Exp. Med. *168*, 687-698.

Miller, J., Selsing, E., and Storb, U. (1982). Structural alterations in J regions of mouse immunoglobulin lambda genes are associated with differential gene expression. Nature *295*, 428-430.

Milne, C.D., Fleming, H.E., Zhang, Y., and Paige, C.J. (2004). Mechanisms of selection mediated by interleukin-7, the preBCR, and hemokinin-1 during B-cell development. Immunol Rev *197*, 75-88.

Miyake, K., Medina, K., Ishihara, K., Kimoto, M., Auerbach, R., and Kincade, P.W. (1991). A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture 1. J. Cell Biol. *114*, 557-565.

Mogensen, T.H. and Paludan, S.R. (2005). Reading the viral signature by Toll-like receptors and other pattern recognition receptors. J. Mol. Med. *83*, 180-192.

Molina, H., Holers, V.M., Li, B., Fung, Y., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R.W., and Chaplin, D.D. (1996). Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc. Natl. Acad. Sci. U. S. A *93*, 3357-3361.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869-877.

Montecino-Rodriguez, E. and Dorshkind, K. (2002). Identification of B/macrophage progenitors in adult bone marrow. Semin. Immunol *14*, 371-376.

Morris, D.L. and Rothstein, T.L. (1993). Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. J. Exp. Med. *177*, 857-861.

Mostoslavsky, R., Alt, F.W., and Rajewsky, K. (2004). The lingering enigma of the allelic exclusion mechanism. Cell *118*, 539-544.

Murasawa, M., Okada, S., Obata, S., Hatano, M., Moriya, H., and Tokuhisa, T. (2002). GL7 defines the cycling stage of pre-B cells in murine bone marrow. Eur. J. Immunol *32*, 291-298.

Nemazee, D., Martensson, A., and Verkoczy, L. (2002). Haplotype exclusion and receptor editing: irreconcilable differences? Semin. Immunol *14*, 191-198.

Nemazee, D.A. and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562-566.

Neuberger, M.S., Di Noia, J.M., Beale, R.C., Williams, G.T., Yang, Z., and Rada, C. (2005). Somatic hypermutation at A.T pairs: polymerase error versus dUTP incorporation. Nat. Rev Immunol *5*, 171-178.

Nitschke, L., Carsetti, R., Ocker, B., Kohler, G., and Lamers, M.C. (1997). CD22 is a negative regulator of B-cell receptor signalling. Curr. Biol. *7*, 133-143.

Norsworthy, P.J., Taylor, P.R., Walport, M.J., and Botto, M. (1999). Cloning of the mouse homolog of the 126-kDa human C1q/MBL/SP-A receptor, C1qR(p). Mamm. Genome *10*, 789-793.

Norvell, A., Mandik, L., and Monroe, J.G. (1995). Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J. Immunol *154*, 4404-4413.

O'Keefe, T.L., Williams, G.T., Davies, S.L., and Neuberger, M.S. (1996). Hyperresponsive B cells in CD22-deficient mice. Science *274*, 798-801.

Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., and Zinkernagel, R.M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. Science *286*, 2156-2159.

Ohmura, K., Kawamoto, H., Fujimoto, S., Ozaki, S., Nakao, K., and Katsura, Y. (1999). Emergence of T, B, and myeloid lineage-committed as well as multipotent hemopoietic progenitors in the aorta-gonad-mesonephros region of day 10 fetuses of the mouse. J. Immunol. *163*, 4788-4795.

Ohnishi, K. and Melchers, F. (2003). The nonimmunoglobulin portion of lambda5 mediates cell-autonomous pre-B cell receptor signaling. Nat. Immunol *4*, 849-856.

Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. (1992). A transgenic model of autoimmune hemolytic anemia. J. Exp. Med. *175*, 71-79.

Oltz, E.M., Yancopoulos, G.D., Morrow, M.A., Rolink, A., Lee, G., Wong, F., Kaplan, K., Gillis, S., Melchers, F., and Alt, F.W. (1992). A novel regulatory myosin light chain gene distinguishes pre-B cell subsets and is IL-7 inducible. EMBO J. *11*, 2759-2767.

Ostermann, G., Weber, K.S., Zernecke, A., Schroder, A., and Weber, C. (2002). JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes
1. Nat. Immunol *3*, 151-158.

Otipoby, K.L., Andersson, K.B., Draves, K.E., Klaus, S.J., Farr, A.G., Kerner, J.D., Perlmutter, R.M., Law, C.L., and Clark, E.A. (1996). CD22 regulates thymus-independent responses and the lifespan of B cells. Nature *384*, 634-637.

Ovary, Z. (1982). Recent insight into class-specific properties of murine immunoglobulins obtained with the help of monoclonal antibodies. Int. Arch. Allergy Appl. Immunol *69*, 385-392.

Pan, C., Baumgarth, N., and Parnes, J.R. (1999). CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. Immunity. *11*, 495-506.

Papayannopoulou, T. and Craddock, C. (1997). Homing and trafficking of hemopoietic progenitor cells
1. Acta Haematol. *97*, 97-104.

Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., and Chan, A.C. (1999). Requirement for B cell linker protein (BLNK) in B cell development. Science *286*, 1949-1954.

Parker, D.C. (1993). The functions of antigen recognition in T cell-dependent B cell activation. Semin. Immunol *5*, 413-420.

Pecquet, S.S., Ehrat, C., and Ernst, P.B. (1992). Enhancement of mucosal antibody responses to Salmonella typhimurium and the microbial hapten phosphorylcholine in mice with X-linked immunodeficiency by B-cell precursors from the peritoneal cavity. Infect. Immun. *60*, 503-509.

Pelanda, R., Braun, U., Hobeika, E., Nussenzweig, M.C., and Reth, M. (2002). B cell progenitors are arrested in maturation but have intact VDJ recombination in the absence of Ig-alpha and Ig-beta. J. Immunol *169*, 865-872.

Petrenko, O., Beavis, A., Klaine, M., Kittappa, R., Godin, I., and Lemischka, I.R. (1999). The molecular characterization of the fetal stem cell marker AA4. Immunity. *10*, 691-700.

Qian, Y., Santiago, C., Borrero, M., Tedder, T.F., and Clarke, S.H. (2001). Lupus-specific antiribonucleoprotein B cell tolerance in nonautoimmune mice is maintained by differentiation to B-1 and governed by B cell receptor signaling thresholds. J. Immunol. *166*, 2412-2419.

Reid, R.R., Prodeus, A.P., Khan, W., Hsu, T., Rosen, F.S., and Carroll, M.C. (1997). Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. J. Immunol *159*, 970-975.

Reth, M. (2001). Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. Trends Immunol. *22*, 356-360.

Reth, M., Gehrmann, P., Petrac, E., and Wiese, P. (1986). A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. Nature *322*, 840-842.

Rickert, R.C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature *376*, 352-355.

Rickert, R.C., Roes, J., and Rajewsky, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res. *25*, 1317-1318.

Rolink, A., Kudo, A., Karasuyama, H., Kikuchi, Y., and Melchers, F. (1991). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J. *10*, 327-336.

Rolink, A. and Melchers, F. (1991). Molecular and cellular origins of B lymphocyte diversity. Cell *66*, 1081-1094.

Rolink, A.G., ten Boekel, E., Yamagami, T., Ceredig, R., Andersson, J., and Melchers, F. (1999). B cell development in the mouse from early progenitors to mature B cells. Immunol. Lett. *68*, 89-93.

Rolink, A.G., Tschopp, J., Schneider, P., and Melchers, F. (2002). BAFF is a survival and maturation factor for mouse B cells. Eur. J. Immunol. *32*, 2004-2010.

Roschenthaler, F., Hameister, H., and Zachau, H.G. (2000). The 5' part of the mouse immunoglobulin kappa locus as a continuously cloned structure. Eur. J. Immunol. *30*, 3349-3354.

Ross, D.A., Wilson, M.R., Miller, N.W., Clem, L.W., and Warr, G.W. (1998). Evolutionary variation of immunoglobulin mu heavy chain RNA processing pathways: origins, effects, and implications. Immunol Rev *166*, 143-151.

Rothstein, T.L. (2002). Cutting edge commentary: two B-1 or not to be one. J. Immunol *168*, 4257-4261.

Rothstein, T.L., Fischer, G.M., Tanguay, D.A., Pavlovic, S., Colarusso, T.P., Gerstein, R.M., Clarke, S.H., and Chiles, T.C. (2000). STAT3 activation, chemokine receptor expression, and cyclin-Cdk function in B-1 cells. Curr. Top. Microbiol. Immunol *252*, 121-130.

Rothstein, T.L. and Kolber, D.L. (1988a). Anti-Ig antibody inhibits the phorbol ester-induced stimulation of peritoneal B cells. J. Immunol *141*, 4089-4093.

Rothstein, T.L. and Kolber, D.L. (1988b). Peritoneal B cells respond to phorbol esters in the absence of co-mitogen. J. Immunol *140*, 2880-2885.

Rothstein, T.L., Kolber, D.L., Murphy, T.P., and Cohen, D.P. (1991). Induction of phorbol ester responsiveness in conventional B cells after activation via surface Ig. J. Immunol *147*, 3728-3735.

Sanchez, P., Marche, P.N., Rueff-Juy, D., and Cazenave, P.A. (1990). Mouse V lambda x gene sequence generates no junctional diversity and is conserved in mammalian species. J. Immunol. *144*, 2816-2820.

Sandel, P.C. and Monroe, J.G. (1999). Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter
1. Immunity *10*, 289-299.

Sato, S., Miller, A.S., Inaoki, M., Bock, C.B., Jansen, P.J., Tang, M.L., and Tedder, T.F. (1996a). CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity. *5*, 551-562.

Sato, S., Ono, N., Steeber, D.A., Pisetsky, D.S., and Tedder, T.F. (1996b). CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. J. Immunol *157*, 4371-4378.

Schlegel, P.G., Vaysburd, M., Chen, Y., Butcher, E.C., and Chao, N.J. (1995). Inhibition of T cell costimulation by VCAM-1 prevents murine graft-versus-host disease across minor histocompatibility barriers. J. Immunol *155*, 3856-3865.

Schlesinger, M. (1965). Spontaneous occurrence of autoantibodies cytotoxic to thymus cells in the sera of mice of the 129 strain. Nature *207*, 429-430.

Schlissel, M.S. (2004). Regulation of activation and recombination of the murine Igkappa locus. Immunological Reviews *200*, 215-223.

Schmidt, K.N., Hsu, C.W., Griffin, C.T., Goodnow, C.C., and Cyster, J.G. (1998). Spontaneous follicular exclusion of SHP1-deficient B cells is conditional on the presence of competitor wild-type B cells. J. Exp. Med. *187*, 929-937.

Schweitzer, K.M., Drager, A.M., van, d., V, Thijsen, S.F., Zevenbergen, A., Theijsmeijer, A.P., van der Schoot, C.E., and Langenhuijsen, M.M. (1996). Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues
1. Am. J. Pathol. *148*, 165-175.

Selsing, E., Miller, J., Wilson, R., and Storb, U. (1982). Evolution of mouse immunoglobulin lambda genes. Proc. Natl. Acad. Sci. U. S A *79*, 4681-4685.

Shaw, P.X., Horkko, S., Chang, M.K., Curtiss, L.K., Palinski, W., Silverman, G.J., and Witztum, J.L. (2000). Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J. Clin. Invest *105*, 1731-1740.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., and . (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855-867.

Solvason, N., Lehuen, A., and Kearney, J.F. (1991). An embryonic source of Ly1 but not conventional B cells. Int. Immunol *3*, 543-550.

Spanopoulou, E., Roman, C.A., Corcoran, L.M., Schlissel, M.S., Silver, D.P., Nemazee, D., Nussenzweig, M.C., Shinton, S.A., Hardy, R.R., and Baltimore, D. (1994). Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. Genes Dev. *8*, 1030-1042.

Su, S.D., Ward, M.M., Apicella, M.A., and Ward, R.E. (1991). The primary B cell response to the O/core region of bacterial lipopolysaccharide is restricted to the Ly-1 lineage. J. Immunol *146*, 327-331.

Su, T.T., Guo, B., Wei, B., Braun, J., and Rawlings, D.J. (2004). Signaling in transitional type 2 B cells is critical for peripheral B-cell development. Immunol Rev *197*, 161-178.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., and Koyasu, S. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science *283*, 390-392.

Taki, S., Schmitt, M., Tarlinton, D., Forster, I., and Rajewsky, K. (1992). T cell-dependent antibody production by Ly-1 B cells. Ann. N. Y. Acad. Sci. *651*, 328-335.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K., and Tybulewicz, V.L. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature *374*, 467-470.

Taylor, P.R., Gordon, S., and Martinez-Pomares, L. (2005). The mannose receptor: linking homeostasis and immunity through sugar recognition. Trends Immunol *26*, 104-110.

Tedford, K., Nitschke, L., Girkontaite, I., Charlesworth, A., Chan, G., Sakk, V., Barbacid, M., and Fischer, K.D. (2001). Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. Nat. Immunol. *2*, 548-555.

Terry, R.W., Kwee, L., Baldwin, H.S., and Labow, M.A. (1997). Cre-mediated generation of a VCAM-1 null allele in transgenic mice
1. Transgenic Res. *6*, 349-356.

Tonegawa, S., Brack, C., Hozumi, N., and Pirrotta, V. (1978a). Organization of immunoglobulin genes. Cold Spring Harb. Symp. Quant. Biol. *42 Pt 2*, 921-931.

Tonegawa, S., Maxam, A.M., Tizard, R., Bernard, O., and Gilbert, W. (1978b). Sequence of a mouse germ-line gene for a variable region of an immunoglobulin light chain. Proc. Natl. Acad. Sci. U. S A *75*, 1485-1489.

Tumang, J.R., Hastings, W.D., Bai, C., and Rothstein, T.L. (2004). Peritoneal and splenic B-1 cells are separable by phenotypic, functional, and transcriptomic characteristics. Eur. J. Immunol *34*, 2158-2167.

Verthelyi, D. (2001). Sex hormones as immunomodulators in health and disease. Int. Immunopharmacol. *1*, 983-993.

Viau, M. and Zouali, M. (2005). B-lymphocytes, innate immunity, and autoimmunity. Clin. Immunol *114*, 17-26.

Vieira, P. and Rajewsky, K. (1988). The half-lives of serum immunoglobulins in adult mice
2. Eur. J. Immunol *18*, 313-316.

Wang, D., Feng, J., Wen, R., Marine, J.C., Sangster, M.Y., Parganas, E., Hoffmeyer, A., Jackson, C.W., Cleveland, J.L., Murray, P.J., and Ihle, J.N. (2000). Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity. *13*, 25-35.

Wang, H. and Clarke, S.H. (2004a). Positive selection focuses the VH12 B-cell repertoire towards a single B1 specificity with survival function. Immunol Rev *197*, 51-59.

Wang, H. and Clarke, S.H. (2004b). Regulation of B-cell development by antibody specificity. Curr. Opin. Immunol. *16*, 246-250.

Wang, J.H., Avitahl, N., Cariappa, A., Friedrich, C., Ikeda, T., Renold, A., Andrikopoulos, K., Liang, L., Pillai, S., Morgan, B.A., and Georgopoulos, K. (1998). Aiolos regulates B cell activation and maturation to effector state. Immunity. *9*, 543-553.

Wasserman, R., Li, Y.S., Shinton, S.A., Carmack, C.E., Manser, T., Wiest, D.L., Hayakawa, K., and Hardy, R.R. (1998). A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. J. Exp. Med. *187*, 259-264.

Weiss, S., Meyer, J., and Wabl, M.R. (1985). V lambda 2 rearranges with all functional J lambda segments in the mouse. Eur. J. Immunol *15*, 765-768.

Weiss, S. and Wu, G.E. (1987). Somatic point mutations in unrearranged immunoglobulin gene segments encoding the variable region of lambda light chains. EMBO J. *6*, 927-932.

Wellicome, S.M., Kapahi, P., Mason, J.C., Lebranchu, Y., Yarwood, H., and Haskard, D.O. (1993). Detection of a circulating form of vascular cell adhesion molecule-1: raised levels in rheumatoid arthritis and systemic lupus erythematosus
1. Clin. Exp. Immunol *92*, 412-418.

Wetzel, G.D. (1989). Interleukin 5 regulation of peritoneal Ly-1 B lymphocyte proliferation, differentiation and autoantibody secretion. Eur. J. Immunol *19*, 1701-1707.

Winkler, T.H., Rolink, A., Melchers, F., and Karasuyama, H. (1995). Precursor B cells of mouse bone marrow express two different complexes with the surrogate light chain on the surface. Eur. J. Immunol. *25*, 446-450.

Wognum, A.W., Eaves, A.C., and Thomas, T.E. (2003). Identification and isolation of hematopoietic stem cells. Arch. Med. Res. *34*, 461-475.

Wong, P. and Pamer, E.G. (2003). CD8 T cell responses to infectious pathogens. Annu. Rev Immunol *21*, 29-70.

Woodland, D.L. and Dutton, R.W. (2003). Heterogeneity of CD4(+) and CD8(+) T cells. Curr. Opin. Immunol *15*, 336-342.

Woodland, R.T. and Schmidt, M.R. (2005). Homeostatic proliferation of B cells. Semin. Immunol *17*, 209-217.

Wortis, H.H., Teutsch, M., Higer, M., Zheng, J., and Parker, D.C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. Proc. Natl. Acad. Sci. U. S A *92*, 3348-3352.

Xia, Y.F., Liu, L.P., Zhong, C.P., and Geng, J.G. (2001). NF-kappaB activation for constitutive expression of VCAM-1 and ICAM-1 on B lymphocytes and plasma cells. Biochem. Biophys. Res. Commun. *289*, 851-856.

Xu, H., Li, H., Suri-Payer, E., Hardy, R.R., and Weigert, M. (1998). Regulation of anti-DNA B cells in recombination-activating gene-deficient mice. J. Exp. Med. *188*, 1247-1254.

Yother, J., Forman, C., Gray, B.M., and Briles, D.E. (1982). Protection of mice from infection with Streptococcus pneumoniae by anti-phosphocholine antibody. Infect. Immun. *36*, 184-188.

Youinou, P., Mackenzie, L., Katsikis, P., Merdrignac, G., Isenberg, D.A., Tuaillon, N., Lamour, A., Le Goff, P., Jouquan, J., Drogou, A., and . (1990). The relationship between CD5-expressing B lymphocytes and serologic abnormalities in rheumatoid arthritis patients and their relatives
1. Arthritis Rheum. *33*, 339-348.

Young, W.W., Jr., MacDonald, E.M., Nowinski, R.C., and Hakomori, S.I. (1979). Production of monoclonal antibodies specific for two distinct steric portions of the glycolipid ganglio-N-triosylceramide (asialo GM2). J. Exp. Med. *150*, 1008-1019.

Zaitseva, M.B., Mojcik, C.F., Salomon, D.R., Shevach, E.M., and Golding, H. (1998). Co-ligation of alpha4beta1 integrin and TCR rescues human thymocytes from steroid-induced apoptosis. Int. Immunol *10*, 1551-1561.

7 Abbreviations

AID	activation-induced cytidine deaminase
Ab	antibody
Ag	antigen
AHA	autoimmune hemolytic anemia
ATA	anti-thymocyte antibody
BCR	B cell receptor
BM	bone marrow
bp	base pairs
BrMRBC	bromelain treated mouse red blood cells
C	constant region
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity determining region
CLP	common lymphoid precursor
CMP	common myeloid precursor
CSR	class switch recombination
D	diversity
DC	dendritic cell
DNA	deoxyribonucleic acid
DSB	double strand break
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FL	fetal liver
FO	follicular
FR	framework
h	hour(s)
H chain	heavy chain

HSC	hematopoietic stem cell
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IL	Interleukin
IL7R	Interleukin 7 receptor
J	joining
kb	kilobases
L chain	light chain
LDL	low-density lipoprotein
LPS	lipopolysaccharide
Mb	megabases
MHC	major histocompatibility complex
MPP	mulitpotent progenitor
MRBC	mouse red blood cell
MZ	marginal zone
N nucleotide	non-templated nucleotide
NK cell	natural killer cell
NP	Nitrophenol
P nucleotide	palindromic nucleotide
PALS	periarterial lymphatic sheet
PC	phosphorylcholine
PCR	polymerase chain reaction
Pec	peritoneal cavity or peritoneum
PtC	phosphatidyl choline
Rag	recombination activating gene
RNA	ribonucleic acid
RS sequence	recombination signal sequence
RT	reverse transcription
RT	room temperature
SL	surrogate light chain
SLE	systemic lupus erythematousus

Sp	spleen or splenic
SPF	specific pathogen free
TCR	T cell receptor
TD	T cell dependent
TdT	terminal deoxynucleotidyl transferase
TI	T cell independent
TNP	Trinitrophenol
V	variable

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